THE IMPACT OF REDUCED NEURONAL P75NTR EXPRESSION ON SENSORY NEURON PHENOTYPE AND ASSOCIATED GLIA

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Master's of Science in the Department of Anatomy and Cell Biology

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Abstract

The common neurotrophin receptor, p75NTR, has been implicated in diverse responses of sensory neurons including a role in nociception following nerve injury, suggesting that it may serve a similar role in intact sensory neurons and their satellite glial cells (SGCs). To examine the impact of suppressing neuronal p75NTR expression on known molecular modulators/regulators of the nociceptive state namely, the sodium channels $Na_V1.8$ and $Na_V1.9$, the nerve growth factor receptor TrkA, the potassium channel $K_{ir}4.1$, glial fibrillary acidic protein (GFAP), SGC p75NTR, connexin 43, we intrathecally infused p75NTR anti-sense oligonucleotides (AS OGN), previously shown by Obata et al. (2006) to effectively suppress p75NTR expression in intact neurons. Male, Wistar rats were divided into three groups, receiving either no treatment (non-infused), seven day intrathecal infusion of p75NTR AS OGN or sense control (SC OGN) via an osmotic pump. Serial L4 and L5 DRG sections were processed for immunohistochemistry to detect alterations in $Na_V1.8$, $Na_V1.9$, TrkA, $K_{ir}4.1$, p75NTR, GFAP and connexin-43 protein expression. Sciatic nerve sections were also processed for immunohistochemistry to detect $Na_V1.8$, $Na_V1.9$, TrkA and GFAP protein expression.

Infusion of p75NTR AS OGNs resulted in a significant decrease in neuronal p75NTR expression, however no significant change was observed in neuronal $Na_V1.8$, $Na_V1.9$ or TrkA expression relative to SC OGN treated or non-infused controls. On the contrary, SGC expression of phenotypic markers normally associated with the reactive state that is induced in these cells in response to peripheral nerve axotomy was dramatically altered. More specifically, in response to p75NTR AS OGN infusion, there was a significant increase in SGC protein expression of the cytoskeletal protein GFAP and p75NTR, along with a significant decrease in expression of the inward rectifying potassium channel $K_{ir}4.1$. Preliminary data also revealed this induced reactive

state in SGCs to be associated with an increase in the number of SGCs surrounding individual neurons as well as increased SGC expression of the gap junction protein, connexin 43.

In conclusion, reductions in neuronal p75NTR expression and potentially reduced neurotrophin signaling lead to alterations in neuron/glial or axon/glial communication that results in induction of a reactive phenotype in the associated SGCs. With our ever increasing understanding of the role of SGCs modulating pain states, elucidation of the pathways leading to adoption of pathological phenotypes can help in the identification of novel therapeutic targets.

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AS	Anti-sense oligonucleotides
ATP	Adenosine triphosphate
BDNF	Brain-derived nerve growth factor
Ca^{2+}	Calcium
CCI	Chronic constriction injury
DRG	Dorsal root ganglion
IB4	Isolectin from *Bandeiraea simplicifolia*
ILI	Immunoreactive labeling intensity
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
K^+	Potassium
$K_{ir}4.1$	Inward rectifying potassium channel 4.1
L4	Lumbar segment 4
L5	Lumbar segment 5
mRNA	Messenger ribonucleic acid
Na^+	Sodium
$Na_V1.8$	Voltage-gated sodium channel 1.8
$Na_V1.9$	Voltage-gated sodium channel 1.9
NGF	Nerve growth factor
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5
NSAIDs	Non-steroidal anti-inflammatory drugs
NOS	Nitric oxide synthase
p75NTR	p75 neurotrophin receptor
SC	Sense control oligonucleotides
s.e.m.	Standard error of the mean
SGC	Satellite glial cell
TRPV1	Transient receptor potential vanilloid receptor-1
TrkA	Tropomyosin related kinase type A
TrkB	Tropomyosin related kinase type B
Trk C	Tropomyosin related kinase type C

1. Introduction

Neuropathic pain is a syndrome characterized by chronic spontaneous aberrant neural firing of sensory neurons in the absence of noxious stimuli. This can include amplification of sensation to mechanical stimuli (mechanical allodynia) where input previously perceived as nonnoxious is now noxious and reduced thresholds for thermal stimuli (thermal hyperalgesia). Thus, warmth, cold, light touch or no sensation at all, may be perceived as painful.

In contrast to acute pain that is effectively treated with general analgesics such as opioids, chronic pain is difficult to treat. Current therapies for chronic pain are not adequate and include antidepressants, non-steroidal anti-inflammatory drugs (NSAIDs) and potent opioids. The plethora of treatment options stems mostly from the poorly understood etiology of the neuropathic pain state and the lack of specific drug targets (reviewed in Chen et al., 2004). Elucidating the underlying anatomical, morphological and chemical changes that cause nerves to transition from normal baseline functioning to anomalous, hypersensitive malfunction is of paramount importance. Until these are elucidated, nonspecific systemic treatments that only slightly mitigate the pain syndrome will continue to be used.

1.1 Pain

Pain occurs in response to noxious stimuli. It encourages the organism to withdraw from a sensory experience, to protect damaged tissue and to learn what harmful situations to avoid in the future. It is considered essential to the survival of an organism; individuals with congenital insensitivity to pain usually do not live past childhood

(reviewed in Nagasako et al., 2003). Without the sensation of pain, young children will chew on their own tongue, lips, fingers and will often not be aware of having experienced serious injuries. Some of these genetic mutations are associated with voltage-gated Na^+ ionic channels responsible for propagating action potentials in sensory neurons (reviewed in Lampert et al., 2010). When excessive chemical, mechanical or thermal stimuli are applied to tissue, nociceptive nerve fibers experience a depolarization that initiates action potentials.

Neuropathic pain results from a direct or indirect injury to the nervous system and has been described as 'bad pain' as it does not promote the survival of the organism (reviewed in Iadarola and Caudle, 1997). The nerve fibers are thus inflamed, dysfunctional or injured and correspondingly do not depolarize and propagate action potentials within normal baseline parameters. Injuries to the nerves may occur, for example, as a result of a trauma, infection or as a secondary etiology to many diseases including alcoholism, diabetes, human immunodeficiency virus, multiple sclerosis or herpes zoster (shingles). The symptoms are often described as shooting/burning or as tingling/numbness. These sensations may occur more frequently than normal or may be prolonged due to altered thresholds for stimulation. Eventually, they may occur spontaneously in the absence of stimuli. This altered sensitivity characteristic of neuropathic pain is associated with changes in cell morphology, membrane ion channels such as voltage-gated sodium channels or potassium channels, receptors, proteins, neurotrophins and many other factors.

1.2 Introduction to the nervous system

The nervous system is a complex network of specialized cells and supporting elements that effectively and efficiently transmit signals across parts of the body. These signals are conveyed via action potentials established by ionic gradients across the neuronal membrane. The central nervous system consists of the brain and spinal cord and the peripheral nervous system includes the cranial nerves and spinal nerves. The perception of one's environment relies directly on the afferent component of the nervous system, the sensory system. The somato-sensory system is charged with relaying a variety of sensations such as proprioception, mechanoception and nociception and thus, consists of a variety of neurons. The cell bodies of these neurons are situated in structures located just outside the spinal cord parenchyma called the dorsal root ganglia (DRG) (See Figure 1.1). A single pseudounipolar process extends from the cell body of each neuron, and this branches into two axonal processes: one projects distally to the peripheral tissues and the other branches centrally to terminate within the dorsal horn of the spinal cord or within the brainstem nuclei via the dorsal columns. Sensory information is consequently transmitted from the peripheral tissues to the brainstem and then onwards to the thalamus, cerebellum and cerebral cortex. This directly influences both an organism's perception and response to external stimuli (reviewed in Kandel et al., 2000).

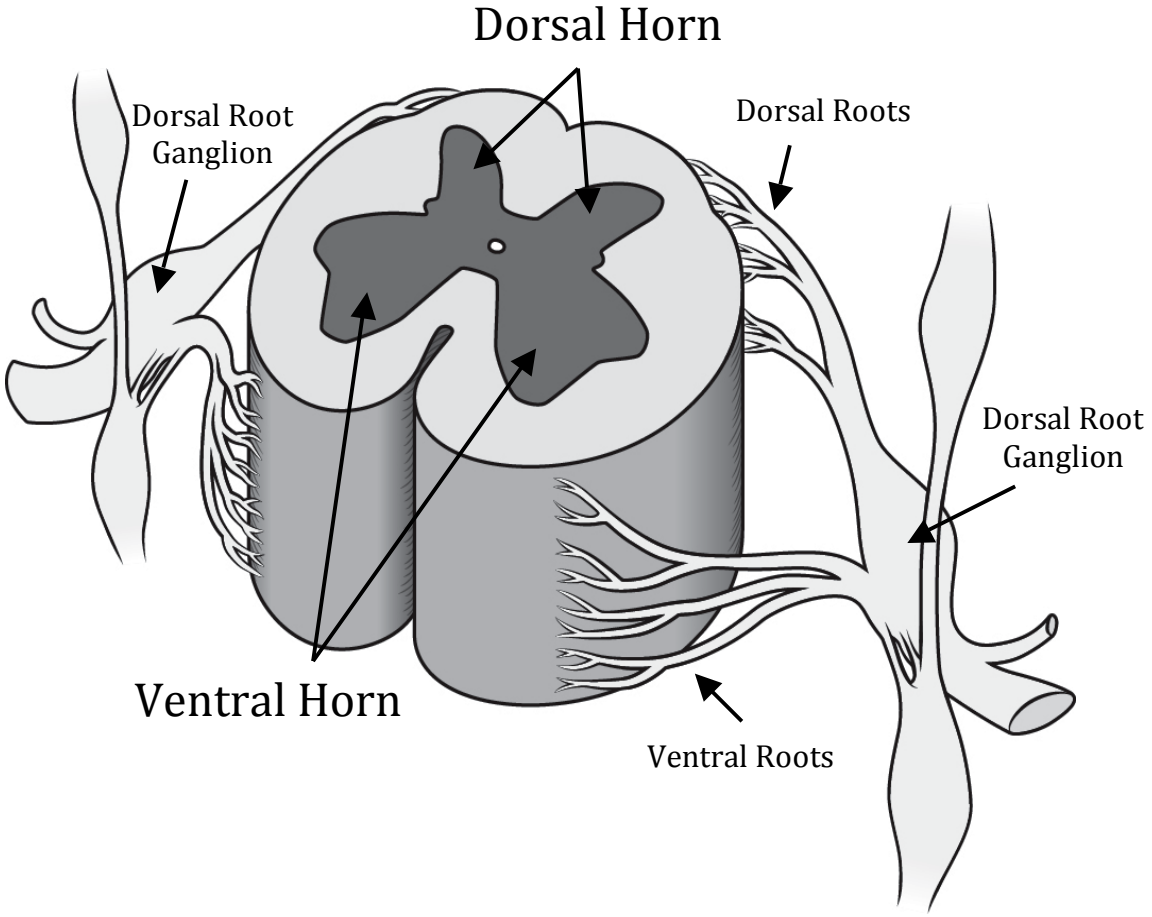

Figure 1.1 The dorsal root ganglia. Sensory information from the peripheral nervous system inputs into the spinal cord. Sensory afferents in the dorsal roots enter into the dorsal/posterior side of the spinal cord, with the pain fibers largely terminating in the region referred to as the dorsal horns, while the motor neuron efferents housed in the region known as the ventral horn, exit the spinal cord on the ventral/anterior side via the ventral roots. The cell bodies of the sensory nerves are located in a structure, just outside the spinal cord, known as the dorsal root ganglion.

1.3 Sensory neurons

1.3.1 Anatomic and physiological properties

The somato-sensory system is comprised of subpopulations of neurons that express different receptors in order to be activated by different types of stimuli. Thus, sensory neurons are commonly classified by the type of stimulus that activates them: proprioceptive, mechanoreceptive or nociceptive. These three types of neurons have significantly different conduction velocities of their action potentials owing to different axonal properties (reviewed in Millan, 1999; Villiere and McLachlan, 1996). Proprioceptive neurons' axons are known as A-α fibers, are large in diameter (12-20 μm), are highly myelinated and have a conduction velocity of 70-120 m/s. Mechanoreceptive neurons' axons known as A-β fibers, are medium in diameter (5-15 μm), are myelinated and have a conduction velocity of 30-80 m/s. Lastly, nociceptive neurons' axons can be subdivided into two groups: the first are known as A-δ fibers, are small in diameter (3-8 μm), are myelinated and have a conduction velocity of 10-30 m/s and the second, known as C fibers, are very small in diameter (0.2-1.5 μm), are unmyelinated and have a conduction velocity of 0.5-2.5 m/s. The cell bodies of the sensory neurons are mostly aggregated along the periphery of the DRG structure with their processes located more medially. In parallel with the diameters of their axons, the cell bodies of the various sensory neurons can roughly be separated by their diameters: proprioceptive neurons are ~40-80 μm, mechanoreceptive are ~25-40 μm and nociceptive neurons are ~10-25 μm, with some overlap between the three (Crowley et al., 1994; Smeyne et al., 1994). For example, there are some small-medium sized cells that are low

threshold mechanoreceptors and some medium-large cells that are nociceptors (Djouhri et al., 2003b; Fang et al., 2002).

1.3.2 Associated glia and sensory neuron/glial communication

The peripheral nervous system contains two well-known types of glial cells, Schwann cells and perineuronal satellite glial cells (SGCs), and a few poorly characterized cells that resemble microglia and less differentiated Schwann cells. A large portion of the Schwann cells envelops the axons of the majority of neurons, producing an insulating protein called myelin, which increases the conduction velocity of action potentials. The SGCs, initially described and somewhat dismissed as a type of Schwann cell, are actually very unique from other glial cells in terms of both their development and the functional structure they form. Multiple SGCs encapsulate the cell body of each sensory neuron within the DRG structure, forming a cellular envelope (reviewed in Hanani, 2005; Pannese, 1981 see Figure 1.2). The barrier they form, usually one to three layers thick (Pannese, 1960), allows for the diffusion of most molecules (Shinder and Devor, 1994), unlike the blood brain barrier, but helps to maintain a homeostatic environment necessary for the passing of action potentials in the sensory neurons (reviewed in Hanani, 2005). This role appears to be vital as cross excitation of action potentials occurring across the DRG because of neural apposition is unlikely because, once matured, less than 2% of neurons are closely apposed (Shinder et al., 1998). Therefore, this substantiates that SGCs may be responsible for propagating excitability in the DRG. Moreover, SGCs contribute to the neuronal homeostatic environment by

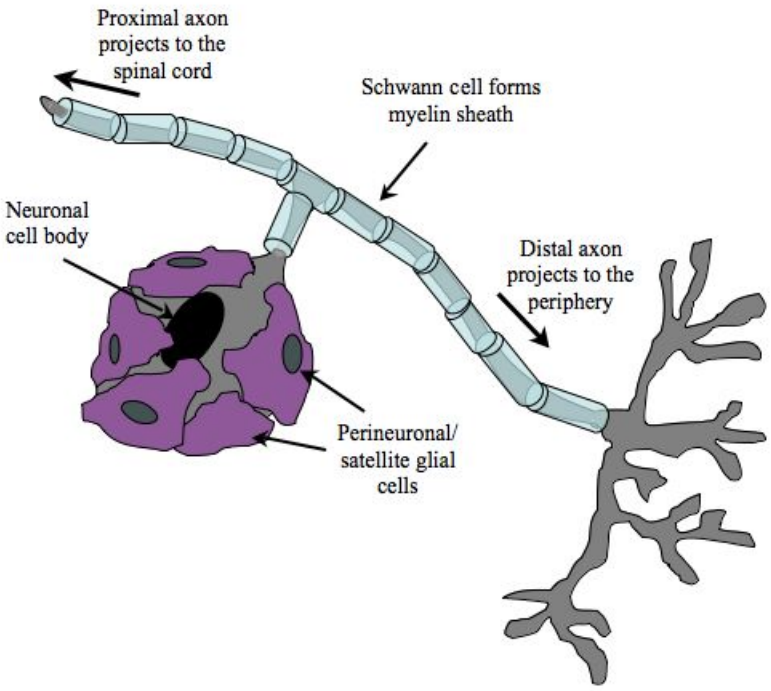

Figure 1.2 Sensory neuron and satellite glial cells. The sensory neuron is pseudo-unipolar in structure. One apparent process extends from the cell body and then branches into the peripheral axon that projects to the periphery of the body and the central axon that projects to the spinal cord and central nervous system. Some of these sensory neurons have myelinated axons (as shown above) and others have unmyelinated axons and are referred to as C-fibers. All of the sensory neuron cell bodies are encapsulated by supporting glials cell unique to the peripheral nervous system, the satellite glial cells (SGCs).

taking up and degrading neurotransmitters (Braun et al., 2004; Keast and Stephensen, 2000).

1.4 Role of neurotrophins in sensory neurons and associated glia with relation to neuropathic pain states

1.4.1 Members of the neurotrophin family

Neurotrophins in the peripheral nervous system modulate and regulate the survival, differentiation, and maintenance of neurons and glia. The mammalian family of neurotrophins consists of nerve growth factor (NGF), brain-derived nerve growth factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). They bind to the tropomyosin-related kinase receptors (Trk) and/or the common neurotrophin receptor, p75NTR (See Figure 1.3), are taken up axonally and transported retrogradely to the cell bodies of the sensory neurons (Curtis et al., 1995). All of the neurotrophins have approximately 50% amino acid homology between them, share similar three-dimensional conformation and are seen as homodimers in their active state. Because of a highly lipophilic center region within the neurotrophins, they are thus capable of forming heterodimers with other neurotrophins (Robinson et al., 1995), although any role for this remains to be elucidated *in vivo*.

Glial derived neurotrophic factor (GDNF), a member of the transforming growth factor β family, also has trophic effects on sensory neurons, despite being structurally different from the neurotrophin family, being in a class of its own. It acts primarily on the ~20% of primarily small sensory neurons that do not express neurotrophin receptors and are often referred to as nonpeptidergic (Henderson et al., 1994; Trupp et al., 1995).

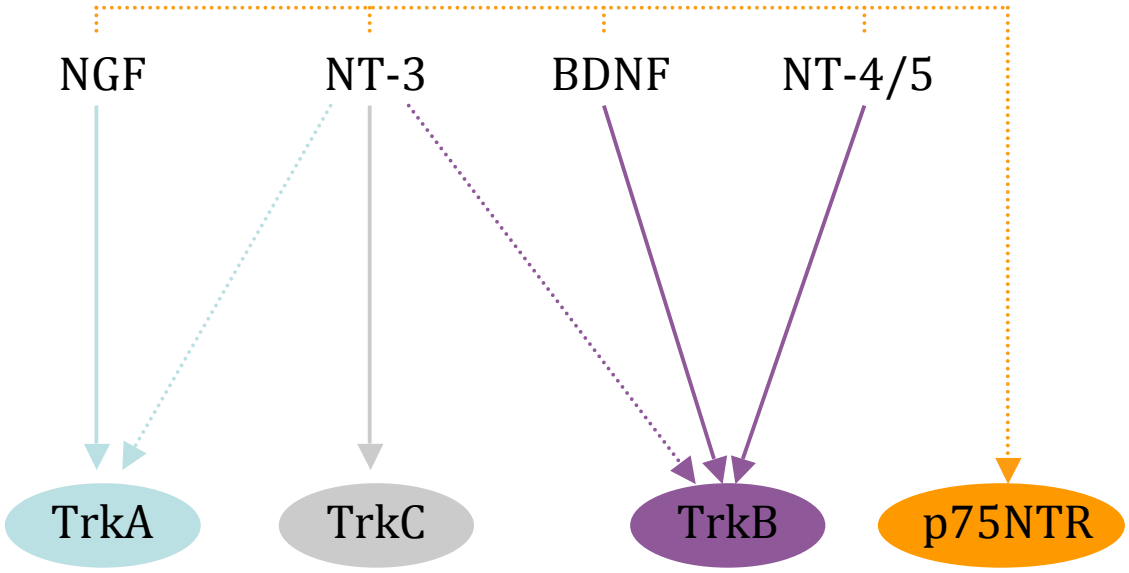

Figure 1.3 Neurotrophin and neurotrophin receptor interactions. Neurotrophins in the peripheral nervous system modulate and regulate the survival, differentiation, and maintenance of neurons and glia. The mammalian family of neurotrophins consists of nerve growth factor (NGF), brain-derived nerve growth factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). They bind to the tropomyosin-related kinase receptors (Trk) with higher affinity (solid arrows) and/or the common neurotrophin receptor, p75NTR, with lower affinity (dashed arrows). NGF binds to TrkA, NT-3 preferentially binds to TrkC but can also bind to TrkA and TrkB, while BDNF and NT-4/5 both bind to TrkB.

NGF was the first neurotrophin to be identified back in the early 1950's by Rita Levi-Montalcini and Viktor Hamburger. It is a 26-kDa nonglycosylated, homodimeric polypeptide and was given its name by Cohen in 1954. It was initially observed in mouse sarcomas and later on in snake venom and the submaxillary glands of mice (Cohen and Levi-Montalcini, 1956; Levi-Montalcini and Cohen, 1960; Levi-Montalcini and Hamburger, 1951). They described it as a growth factor necessary for the survival and outgrowth of sensory and sympathetic neurons but not motor neurons (Levi-Montalcini and Hamburger, 1951). NGF's primary receptor is TrkA located on small nociceptive neurons and p75NTR (Crowley et al., 1994; Kaplan et al., 1991; Smeyne et al., 1994; Squinto et al., 1991).

BDNF was initially identified in 1982 by Barde et al. It was discovered in 1989 that BDNF was structurally similar to NGF (Leibrock et al., 1989). BDNF binds to TrkB receptors located on the medium sized mechanoreceptive neurons and p75NTR (Klein et al., 1991; Mu et al., 1993; Schecterson and Bothwell, 1992; Snider and Wright, 1996; Squinto et al., 1991).

NT-3 was later identified using homology-based approaches to find factors similar to NGF and BDNF (Hohn et al., 1990; Maisonpierre et al., 1990). NT-3 binds preferentially to TrkC receptors located on the large proprioceptive neurons as well as p75NTR (Farinas et al., 1994; Lamballe et al., 1991; Mu et al., 1993; Squinto et al., 1991). It has also been shown that NT-3 can also bind to TrkA and TrkB albeit with a lower affinity than their higher affinity binding neurotrophins, NGF and BDNF respectively (Ip et al., 1993; Ivanisevic et al., 2007; Klein et al., 1991).

NT-4 was first isolated from a xenopus and a viper (Hallbook et al., 1991) and NT-5 from the placenta of a human fetus (Berkemeier et al., 1991). It was later discovered that they are two different species variations of the same neurotrophin now commonly referred to as NT-4/5. NT-4/5 like BDNF, binds to TrkB receptors located on the medium sized mechanoreceptive neurons that also express p75NTR (Berkemeier et al., 1991).

GDNF was first purified in 1993 and it was initially believed that it was a survival factor for only dopaminergic neurons (Lin et al., 1993). GDNF has a strong trophic effect on Schwann cells and sensory neurons (Matheson et al., 1997; Naveilhan et al., 1997).

1.4.2 Sources of neurotrophins for sensory neurons

NGF, NT-3, NT-4/5 and GDNF are derived from the sensory neurons' effector tissues and following binding to, and internalization with their high-affinity Trk receptor, travel to the somata via retrograde transport (Cui et al., 2007; Curtis et al., 1995; Helgren et al., 1997; Ibanez et al., 1993; Matheson et al., 1997; Timmusk et al., 1993). Unlike the other neurotrophins, BDNF is synthesized by some of the DRG neurons. In the intact axonal state, its expression is primarily localized to the small, TrkA expressing, nociceptive population of neurons in the DRG (Karchewski et al., 2002). The SGCs, surrounding the neuronal cell bodies, actually synthesize low levels of NGF, NT-3 and GDNF themselves (Hammarberg et al., 1996; Zhou et al., 1999). Finally, nerve-derived Schwann cells also produce low levels of NGF, BDNF, NT-3, NT-4/5 and GDNF (Hammarberg et al., 1996; Meyer et al., 1992; Ming et al., 1999; Saika et al., 1991; Watabe et al., 1995).

1.4.3 Members of the neurotrophin receptor family

All of the neurotrophins bind to two types of receptors: members of the Trk family of receptor tyrosine kinases and the common neurotrophin receptor, p75NTR. There are three mammalian Trk (tropomyosin-related kinase) receptors: TrkA, TrkB and TrkC. They are transmembrane glycoproteins approximately 140 kD in size (~800 amino acids) (Schneider and Schweiger, 1991) and their functions range from regulating the development, differentiation and survival of neurons to modulating synapse formation and plasticity. There is an approximate 80% amino acid homology in the intracellular tyrosine kinase domains between the Trk receptors and much less in their extracellular domains (approximately 30%) despite having the same subdomain organization with five components: two cysteine-rich motifs, a leucine-rich motif and immunoglobulin-like subdomains Ig-C1 and Ig-C2 (reviewed in Bibel and Barde, 2000). The low homology of the extracellular domains accounts for the specificity and selectivity of the Trk receptors for their ligands. Specific tyrosine residues within the intracellular domains of the Trk receptors can be phosphorylated when neurotrophins bind to the extracellular domain, forming an activation site and allowing for the binding of adaptor proteins (Kaplan and Stephens, 1994).

P75NTR is a member of the tumour necrosis factor receptor family was named as such because of its 75 kDa size (399 amino acids) (Chen et al., 2009). Although it was identified 20 years ago, p75NTR's exact physiological functions are not completely understood. The neurotrophins all bind to p75NTR with less affinity (~10^{-9} M) than the family of Trk receptors (~10^{-11} M) (Rodriguez-Tebar et al., 1990; Rodriguez-Tebar et al., 1992). Despite the similarity in their nanomolar affinity to p75NTR, each neurotrophin

appears to target different residues; each producing different conformational changes (Timm et al., 1994). In addition to signaling independent of the Trk receptors, experimental evidence has implicated p75NTR in regulating Trk signaling (Benedetti et al., 1993; Hempstead et al., 1991; Maliartchouk and Saragovi, 1997). It is believed that these two classes of receptors also exist as one functional unit. P75NTR appears to alter the sub domain of the Trk receptors to reveal an additional binding site for neurotrophins as evidenced by Zaccaro et al. (2001) observing a 10^{-9} M to 10^{-11} M binding affinity increase. For example, TrkC contains a NT-3 binding site that is functionally regulated by p75NTR expression that when activated induces survival but not differentiation (Ivanisevic et al., 2003). The ratio of TrkA to p75NTR appears to be the key in modulating binding affinity and the overall effects of NGF and NT-3 to either of these receptors (Brennan et al., 1999). The TrkA to p75NTR ratio also regulates their own internalization/mobilization rates to the membrane (Makkerh et al., 2005).

1.4.4 Distribution of neurotrophin receptors in sensory ganglia

There exist multiple isoforms of each of the three Trk receptors. TrkB and TrkC are found as either full length or as truncated variants that lack the intracellular tyrosine kinase domain (Klein et al., 1990; Lamballe et al., 1993).

The TrkA receptor is a high-affinity catalytic receptor whose main ligand is NGF and has been identified in ~40-45% of sensory neurons in the lumbar DRG by the presence of its messenger ribonucleic acid (mRNA) that colocalizes with the presence of NGF high-affinity binding sites (Kaplan et al., 1991; Karchewski et al., 1999).

Approximately 89% of TrkA positive neurons are small/medium in size (less than 40 μm in diameter), while the remainder is classified as large (Karchewski et al., 1999).

The TrkB receptor is a high-affinity catalytic receptor whose ligands are BDNF and NT-4/5 and ~33% of lumbar sensory neurons express its mRNA above detection levels (Karchewski et al., 1999; Klein et al., 1991; Squinto et al., 1991). The majority, ~90%, of TrkB positive sensory neurons are medium/large in diameter (Karchewski et al., 1999).

The TrkC receptor is a high affinity catalytic receptor whose ligand is NT-3 and its mRNA has been localized to ~43% of the lumbar sensory neuron population (Karchewski et al., 1999; Lamballe et al., 1991). Of those positive for TrkC, 85% of them are medium/large in diameter (Karchewski et al., 1999).

Evidently, given the percentages of the sensory neuron population positive for the three Trk receptors, some colocalization and trilocalization must exist. It was reported that ~10% coexpress TrkA and TrkB mRNA, ~19% coexpress TrkA and TrkC mRNA, ~18% coexpress TrkB and TrkC and only ~3-4% express all three mRNAs (Karchewski et al., 1999). Truncated forms of TrkB and TrkC, lacking their intracellular catalytic domains, exist in both the central and peripheral nervous systems whose functions are both poorly understood (Klein et al., 1990; Valenzuela et al., 1993).

Approximately 79% of DRG neurons express p75NTR. This expression is across all size ranges and is almost always accompanied by some level of Trk receptor expression (Karchewski et al., 1999). It is highly colocalized with TrkA and positively correlates with the level of TrkA expression (Verge et al., 1992). However, Wright and Snider did not find strong colocalization of p75NTR with all Trk subpopulations as only

8% of TrkB positive sensory neurons express detectable p75NTR mRNA and only about 50% of the TrkCs appeared to do so (Verge et al., 1992; Wright and Snider, 1995). Their lower percentage of overall Trk and p75NTR expression relative to that of the Verge lab (Karchewski et al., 1999) suggest that it may be due to the sensitivity of the technique employed. In addition to neuronal expression, SGCs and Schwann cells are found to express p75NTR (Gai et al., 1996; Zhou et al., 1996).

Distinct from the Trk expressing subpopulations is a population of sensory neurons that do not bind any of the neurotrophins (do not express detectable Trk or p75NTR mRNA), are small/medium in diameter and represent ~20% of sensory neurons (Karchewski et al., 1999). These neurons express the GDNF receptor, which is composed of a ligand-binding subunit known as GDNF receptor α and a signaling tyrosine kinase subunit Ret. The ability to bind isolectin from *griffonia simplicifolia* (IB4) is also another distinguishing characteristic of this subpopulation (Bennett et al., 1998).

1.4.5 Regulation of pain states by neurotrophins

Neuropathic pain states result from two very distinct nerve injuries: compression/constriction and partial nerve severance resulting in inflammation (referred to as a nerve lesion) or complete nerve severance/axotomy (referred to as an axotomy). The type of injury can differentially affect the neurotrophin/neurotrophin receptor levels within both the sensory neuron subpopulations and the glial cells within the peripheral nervous system.

NGF is involved in neuropathic inflammation-associated pain in that it activates the TrkA receptor on nociceptive neurons, modulating signaling and gene expression, promoting hypersensitivity to mechanical and/or thermal stimuli (Jongsma Wallin et al., 2001; Jongsma Wallin et al., 2003; Verge et al., 1995; Verge et al., 1990). A partial nerve lesion upregulates NGF and this leads to the activation and upregulation of receptors including transient receptor potential vanilloid receptor-1 (TRPV1), directly associated with the activation of nociceptive/thermoreceptive sensory neurons (Garcia-Martinez et al., 2002; Ji et al., 2002). Expression of NGF in SGCs increases significantly in the DRG corresponding to a nerve lesion but is still not sufficient to reverse the injury phenotype that appears due to the far more substantial loss of target-derived NGF (Zhou et al., 1999).

BDNF expression rises sharply in ~80% of sensory neurons following a nerve lesion, followed by a reduction in expression in the small, TrkA expressing nociceptive population and an induction in the medium to large, TrkB and/or TrkC expressing population (Karchewski et al., 2002). This phenotypic switch is believed to be necessary in initiating repair programs including remyelination of the nerves. BDNF has been shown to promote myelination during both development and after an injury via the p75NTR present in sensory neurons and Schwann cells *in vitro* and *in vivo* (Chan et al., 2001; Cosgaya et al., 2002; Zhang et al., 2000).

NT-3 has recently been linked to the nociceptive subpopulation of neurons in that it affects a downregulation in the expression of nociception-associated molecules in TrkA-expressing neurons, including that of TrkA. In the injured/inflamed state associated with a chronic constriction injury (CCI) of the nerve where only some of the

nerve fibers are injured, NT-3 can prevent or reverse thermal hypersensitivity and downregulate expression of molecules complicit in this behavior, in contrast to NGF (Gratto and Verge, 2003; Jongsma Wallin et al., 2001; Karchewski et al., 2002; Verge et al., 1996; Wilson-Gerwing et al., 2005). However, no changes in mechanical hypersensitivity were observed with the infusion of NT-3 following a nerve lesion, specifically a CCI (Wilson-Gerwing et al., 2005). In the non-injured/intact state, NT-3 has been shown to downregulate markers associated with nociceptive phenotype including TrkA, voltage-gated Na^+ channels 1.8 and 1.9 ($Na_V1.8$ and $Na_V1.9$), TRPV1 and activated p38 mitogen-activated protein kinase albeit to a far less degree compared to that observed in the CCI state (Gratto and Verge, 2003; Wilson-Gerwing et al., 2005; Wilson-Gerwing et al., 2009). Of note, in the uninjured state, NT-3 fails to affect thermal and/or mechanical thresholds (Wilson-Gerwing et al., 2005). Expression of NT-3 in SGCs increases significantly in the DRG corresponding to a nerve lesion but it is not known if this source results in a direct modulation of the nociceptive phenotype (Zhou et al., 1999).

Currently**,** NT-4/5's role in neuropathic pain states is poorly understood. Its effects are seemingly in parallel to that of BDNF; its injection, or that of BDNF, into the rat hind paw significantly decreased thermal stimulation threshold (Shu et al., 1999). It has been linked to regeneration; NT-4/5 knockout mice were shown to have no enhancement of axonal regeneration with electrical stimulation as compared to wild type mice (English et al., 2007).

GDNF mRNA expression in Schwann cells and SGCs is rapidly upregulated following a nerve injury (Hammarberg et al., 1996). The distal portion of the injured

nerve but not the proximal section or spinal cord expressed higher levels of GDNF-α receptor mRNA. As with NGF and NT-3 (see above) the increased levels of SGC GDNF expression albeit higher, were not sufficient to reverse the injury-induced alteration in neuronal phenotype for this subpopulation – i.e. the decreased IB4 binding (Bennett et al., 1998).

1.4.5.1 Alterations in neurotrophin receptor expression

In parallel with neurotrophin expression levels varying with injury type, neurotrophin receptor expression levels can also be differentially affected by either a partial nerve lesion or complete axotomy. Following a three week lumbar spinal nerve injury, the number of NGF high-affinity binding sites falls drastically, by more than 80% and include a parallel decrease in TrkA and p75NTR mRNA expression (Verge et al., 1989), with mRNA expression levels being 55% below normal levels two weeks post-injury in response to ligation injury (Kuo et al., 2007). With a partial nerve lesion such as a CCI, where only the outside axons are injured leaving the axons in the middle of the nerve intact but exposed to an inflammatory environment, there is a no consistent impact in TrkA expression (Wilson-Gerwing and Verge, 2006). Similar responses with respect to neuronal p75NTR expression were observed by Karchewski et al. (2002) and Kuo et al. (2007) in response to lumbar spinal nerve transection or sciatic nerve ligation injury. In regards to spinal nerve injury-induced downregulation of NGF TrkA and p75NTR receptor expression, this is likely due to the marked decrease in axonal NGF transport from the peripheral effector/target tissues. This is supported by the ability of intrathecally infused NGF to restore NGF TrkA and p75NTR receptor expression to near

pre-injury levels in the chronically injured neurons (Li et al., 2000). The situation is different in the case of a CCI, where the Wallerian-degenerating injured fibers invoke increased expression of NGF in the nerve. As is seen with other partial nerve injury models, the exposure of these uninjured fibers to this inflammatory environment results in increased expression of nociception-associated molecules and instatement of a neuropathic pain state. This is driven largely by NGF influences on these uninjured neurons that are both still connected to an abundant source of NGF in the target tissue and exposed to additional NGF at the site of injury (Fukuoka et al., 2001; Herzberg et al., 1997; Ma and Bisby, 1998; Ma et al., 2010). While not extensively studied, the Verge lab did not note declines in neuronal p75NTR mRNA expression when utilizing the partial nerve injury model CCI (Wilson-Gerwing et al., 2008).

The receptor for BDNF and NT4-5, full length TrkB, can be detected in approximately 33% of adult sensory neurons (Karchewski et al., 1999). In response to lumbar spinal nerve lesion and sciatic nerve injuries, declines in neuronal full length TrkB mRNA expression is evident at time points beyond one day. The slight increase observed at the one day time point is perhaps due to the rapid and transient increase in DRG BDNF expression that is seen at very early time points (Karchewski et al., 2002; Kuo et al., 2007; Obata et al., 2006).

Finally, full length TrkC mRNA is expressed by ~40-45% of DRG neurons (Karchewski et al., 1999). There is discrepancy in how its neuronal expression is altered by nerve injury. The Verge lab observes consistent declines in TrkC expression with spinal nerve injury. This is also observed at the five day injury time point by Obata et al. (2006). This can be reversed in the chronically injured neurons, along with the associated

changes in expression of phenotypic markers for this subpopulation by infusion of exogenous NT-3 two weeks after injury for a one week period (Jongsma Wallin et al., 2001; Karchewski et al., 2002; Verge et al., 1996). As is the case for TrkA, this suggests that disconnection of the axon from target –derived sources of NT-3, likely accounts for the decline in TrkC expression observed following spinal nerve transection and alteration in phenotype observed. These findings are in contrast with those of Kuo et al. (2007), where a notable and sustained increase in TrkC expression was observed in response to sciatic nerve ligation and transection. How the CCI partial nerve injury impacts TrkC expression has not been studied.

1.4.5.2 Alterations in neuronal properties associated with injury/pain states

Maintenance of receptor and channel homeostasis within the neuronal membrane of the sensory system is essential for the proper transmission of proprioceptive, mechanoreceptive or nociceptive information. The ionic gradient necessary for establishing the action potential threshold across the neuronal membrane of nociceptive neurons is reliant on Na^+ channels 1.8 and 1.9.

Expression of the Na^+ channel $Na_V1.8$ appears to be exclusive to the sensory system and specifically localized to the smaller, nociceptive DRG neurons (Djouhri et al., 2003a). Sixty percent of neurons that express $Na_V1.8$ also bind the isolectin *griffonia simplicifolia* (IB4) (that identifies a subpopulation of sensory neurons that is predominantly GDNF-responsive with little or no detectable Trk or p75NTR expression) (Fukuoka et al., 2008) and thus, correspondingly, almost all of the remaining 40% also express p75NTR (Wright and Snider, 1995). $Na_V1.8$ channels are responsible for the

majority of the action potentials and significant changes in their expression levels are believed to contribute to the altered neuronal excitability, playing a role in the aberrant action potential firing experienced leading to chronic pain (Blair and Bean, 2002; Lai et al., 2004; Waxman et al., 1999). It appears that $Na_V1.8$ levels may decrease in the directly injured nerves, small diameter neurons, however, the uninjured adjacent DRG neurons and their axons have increased expression of $Na_V1.8$ and it may contribute to the aberrant action potential activity seen following nerve lesion (Wang et al., 2011).

$Na_V1.9$ is expressed in pain-signalling, smaller DRG neurons (Wang et al., 2011). Within the DRG, 62% of neurons that express $Na_V1.9$ are also IB4-positive (Fukuoka et al., 2008). Clinically, the use of Na^+ channel blockers such as Lidocaine® have been shown to provide some relief of neuropathic pain syndromes (Priest and Kaczorowski, 2007).

$Na_V1.9$ channels are also highly colocalized with TRPV1 and purinergic P_2X_3 receptors (Amaya et al., 2006). These receptors are directly associated with the activation of pain sensations. TRPV1 is highly expressed in both the Trk-A positive subpopulation and the IB4 positive subpopulation of the DRG (Helliwell et al., 1998; Michael and Priestley, 1999) and is activated by heat greater than 43 ^oC, low pH, exogenous compounds including capsaicin associated with spiciness from chili peppers and venoms from jellyfish and spiders, as well as endogenous compounds NGF and anandamide (reviewed in Clapham et al., 2005; Szallasi et al., 2007). The P_2X_3 receptor is an ATP specific ligand-gated ion channel and exists as a homomeric form, P_2X_3, localized to smaller DRG neurons and as a heteromeric form, $P_2X_{2/3}$, localized to medium-large DRG neurons (Kage et al., 2002). The activation of P_2X_3 is believed to

contribute to acute nociception. It is downregulated in damaged peripheral nerves and upregulated in the adjacent uninjured nerves with a partial nerve injury (Tsuzuki et al., 2001) and it is downregulated with an axotomy (Bradbury et al., 1998). On the other hand, the activation of $P_2X_{2/3}$ is believed to drive longer-lasting nociception as its levels within the DRG are not altered following nerve ligation (Kage et al., 2002).

1.4.5.3 Alterations in perineuronal cell phenotype associated with injury/pain states

Glial cells within the central and peripheral nervous systems are associated with maintaining neuronal homeostatic function of the neuronal network. With injury, these glial cells may contribute to the production and the development of chronic pain. In the case of peripheral nerve injuries, the SGCs, unique to the sensory and autonomic systems, are optimally positioned to affect neuronal excitability, tightly surrounding the cell bodies of the sensory neurons within the dorsal root ganglia. With injury, SGCs become highly activated, proliferate and have increased coupling amongst themselves (Dublin and Hanani, 2007; Lu and Richardson, 1991). They correspondingly have increased expression of glial fibrillary acidic protein (GFAP), p75NTR, GDNF, NGF, NT-3, connexin 43, a gap junction protein, and decreased expression of inward rectifying K^+ channel 1.4 ($K_{ir}4.1$) (Guo et al., 2007; Ohara et al., 2008; Tang et al., 2010; Xian and Zhou, 1999; Zhou et al., 1996). Schwann cells also contribute to regeneration following an injury by migrating across the nerve gap, forming a continuous structure to guide axonal regrowth (Torigoe et al., 1996).

GFAP is the predominant constituent of the cytoskeleton of many glial cells. It is an intermediate filament protein and is believed to contribute to the mechanical strength

of the cells, helping to maintain their shape. It has long been established in astrocytes of the central nervous system, however it was only identified in 1984 by Jessen et al. in Schwann cells and satellite cells in both the sensory and autonomic ganglia of the peripheral nervous system. Increased GFAP expression is commonly associated with a transition to a 'reactive state' of the glial cells in question. In the DRG, SGCs express very low levels of GFAP under normal conditions (Vit et al., 2006). Following an injury or chronic inflammation, GFAP levels steadily increase until most SGCs are GFAP immunoreactive (Siemionow et al., 2009). An increase in fibrils within the cytoplasm of SGCs following nerve injury had been described as long ago as 1967 by Leech.

After a chemical or mechanical insult, it has been reported that SGCs surrounding sensory neurons undergo division and proliferate as evidenced by an increased concentration of mitotic figures observed at the electron microscope level (Hiura and Ishizuka, 1989; Humbertson et al., 1969) and the presence of BrdU positive cells surrounding the sensory neuron cell bodies (Cecchini et al., 1999; Elson et al., 2004; Elson et al., 2003). In addition to a proliferation in the number of SGCs after a nerve injury, SGCs are also more coupled amongst themselves, postulated to be a result of an increase in gap junction concentration (Ledda et al., 2009; Zhang et al., 2009). Connexin 43 is a subunit of gap junctions in SGCs, and is not found in the sensory neurons (Ohara et al., 2008). Functionally, gap junctions are involved in the diffusion of ionic currents, including K^+, amongst SGCs (Huang et al., 2005; Konishi, 1996). Thus, connexin 43 expression levels indirectly affect neuronal excitability. The extent of their influence on neuronal function was demonstrated by Vit et al. (2006) by introducing RNA interference specific to connexin 43 to the trigeminal ganglion in rats. This resulted in the

development of spontaneous pain-like behaviour and the return of connexin 43 expression to baseline levels subsequently restored normal behaviour.

$K_{ir}4.1$ is highly expressed in glial cells of the central nervous system. It was recently reported that it is also highly expressed in SGCs (Vit et al., 2006). It is believed that these channels contribute to the hyperpolarized resting membrane potential of SGCs and help regulate the conductory extracellular K^+ levels (Kofuji and Newman, 2004; Olsen and Sontheimer, 2008). These roles are considered vital as buffering is necessary to maintain optimal extracellular K^+ levels for neuronal activity (Kofuji and Newman, 2004). $K_{ir}4.1$ has recently been inversely associated with neuropathic pain. SGCs post-injury have a lower expression of $K_{ir}4.1$ compared to pre-injury levels (Ohara et al., 2009). For example, a chronic constriction injury of the trigeminal nerve, a model of facial pain, resulted in downregulation of $K_{ir}4.1$ levels in trigeminal SGCs. By decreasing $K_{ir}4.1$ expression in rat trigeminal SGCs with small interfering RNAs, facial neuropathic pain-like behaviours were observed by both Ohara et al. (2008) and Vit et al. (2008). These robust findings are not surprising given that the $K_{ir}4.1$ channel appears to be the principal regulator of SGC K^+ levels. The partial and complete genetic ablation of the $K_{ir}4.1$ gene lead to a halving and almost complete loss of the inward K^+ respectively (Tang et al., 2010).

1.4.5.4 Involvement of the common neurotrophin receptor, p75NTR, in neuropathic pain states

Within the organism, expression levels of p75NTR tend to be high during development, taper during adulthood and increase dramatically during pathological states

(Chao, 2003). In terms of the sensory neuron populations, after a nerve axotomy, p75NTR is dramatically downregulated. This reduction can be reversed with the infusion of NGF, however only in the subpopulation expressing TrkA (Verge et al., 1992). Following a nerve lesion, p75NTR expression decreases in the injured primary sensory neurons (Verge et al., 1992), however increased expression is observed in their uninjured primary sensory neighbours and in the surrounding SGCs (Obata et al., 2006). Comparatively, it was reported by Obata et al. (2006) that an increase in the immunoreactivity of p75NTR was observed in SGCs in the DRG, particularly around larger-sized neurons, following lumbar segment 5 (L5) nerve ligation injury. However, this glial p75NTR upregulation was not observed in the adjacent non-injured lumbar segment 4 (L4) ganglia. The application of a p75NTR inhibitory antibody to a pinched sciatic nerve animal model was sufficient to suppress neuronal expression of calcitonin gene-related peptide and p75NTR and significantly decrease mechanical allodynia (Fukui et al., 2010).

In addition to the neurotrophins binding to either p75NTR or the Trk receptors, they can bind to a p75NTR/Trk complex. Numerous reports describe either an allosteric or cooperative interaction between p75NTR and Trk (Bamji et al., 1998; Bibel et al., 1999; Brennan et al., 1999; MacPhee and Barker, 1997; Maliartchouk and Saragovi, 1997). Investigations regarding the p75NTR/TrkA complex binding its NGF ligand currently dominate the literature. A structural study has revealed, based on the resulting crystal formation that the NGF dimer changes conformation after binding to p75NTR to form two NGF monomers with one p75NTR molecule between them (He and Garcia, 2004) in contrast to the 2:2 stoichiometry of the NGF/TrkA complex without p75NTR.

This 2:1 NGF/p75NTR complex offers the possibility of creating a three molecule complex with TrkA. Thus, the relative expression levels of NGF, p75NTR and TrkA would determine the likelihood of creating the various complexes and regulating downstream signaling. The ratio of p75NTR to TrkA appears to be the key in modulating binding affinity and the overall effects of NGF and NT-3 to either of these receptors (Brennan et al., 1999). The p75NTR to TrkA ratio also regulates internalization/mobilization rates of these proteins to the membrane (Makkerh et al., 2005).

1.5 An overview of the mechanism of action of anti-sense oligonucleotides

Anti-sense technology was first used in 1977 by Paterson et al. It consists of a single stranded exact mRNA sequence usually between 12 and 30 chemically stabilized oligonucleotides complimentary to a specific protein which blocks the translation of that protein's mRNA within the cytoplasm (Pestka, 1992; See Figure 1.4). Manufactured antisense oligonucleotides (AS) are optimized to bind the target mRNA as efficiently as possible while minimizing non-specific binding to other mRNA and proteins. Uptake into the cells *in vivo* is associated with the transporter, p80, a transporter for negatively charged macromolecules (the oligonucleotide backbone of the AS is anionic), in addition to passive diffusion/endocytosis into the cells (Biognostik). Modern AS are much more efficient and nuclease-resistant *in vivo,* owing to chemical modifications including the addition of phosphorothioate stabilizing bonds (White et al., 2009). Significant specific protein production decrease *in vivo* is dependent on the route of administration, dosage and tissue uptake.

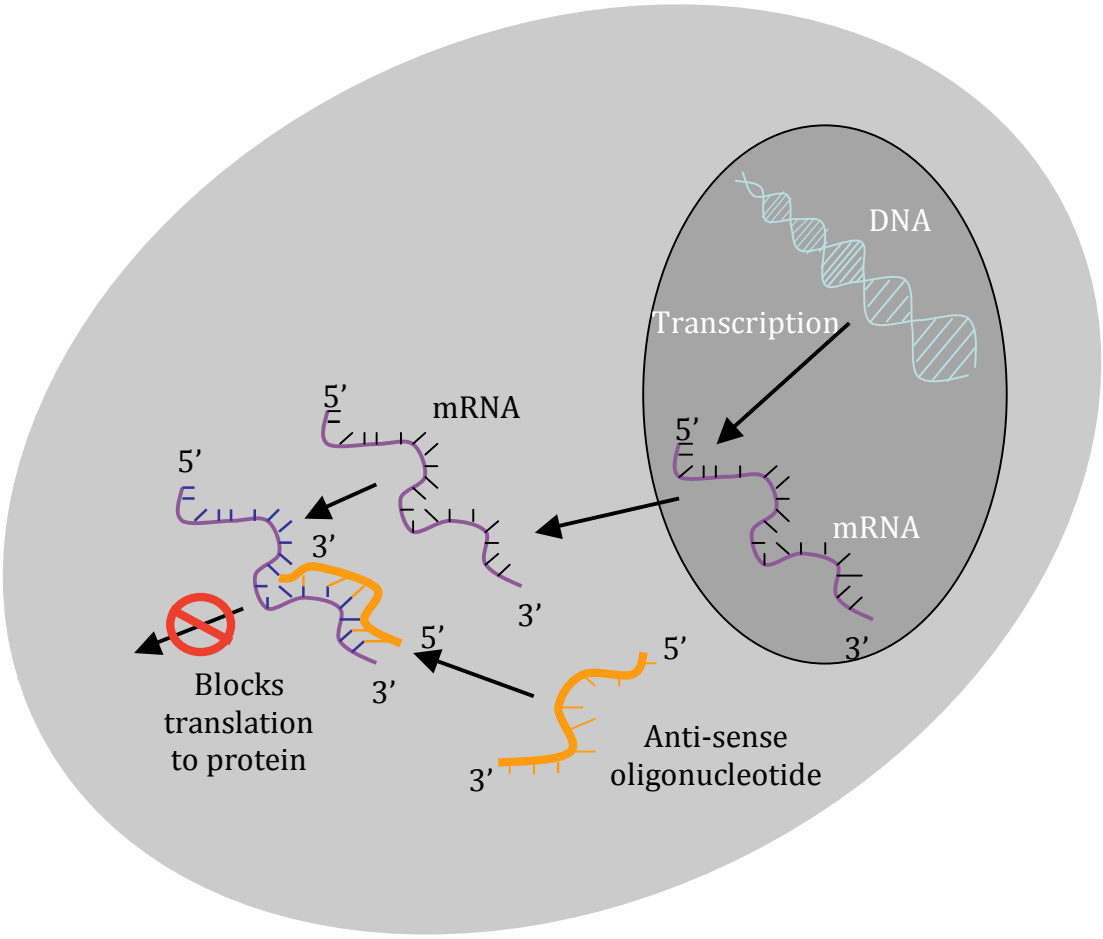

Figure 1.4 Mechanism of action of anti-sense technology. Messenger RNA (mRNA) is transcribed from DNA in the nucleus. It is then transported to the cytoplasm of the cell to be translated to a protein. However, with the introduction of an anti-sense sequence (AS) (ie. one complementary to 15 bases of p75NTR mRNA that typically includes the initiation site), the p75NTR mRNA is prevented from undergoing translation, because the translational machinery cannot access the initiation site. This technique requires an adequate and continuous infusion of the AS to effectively block a significant portion of the p75NTR mRNA from being translated by each cell. Thus, it only significantly blocks p75NTR protein production for the duration of the lifetime of the AS.

In order to verify the uptake pattern of the infused substance, a fluorescein-tagged copy of the AS is infused and visualized within the cytoplasm of the target cells at different timelines. Control oligonucleotides, also known as sense control (SC), consist of random oligonucleotide sequences that are nonhomologous to any known sequences (verified on the GenBank database) and are used to control for non-specific binding of the AS or the phosphorothioates to any proteins and substance-related side effects. They are designed to contain a similar ratio of guanine-cytosine/uracil-adenine base pairs and be of a similar length.

This AS technology has previously been used by Barclay et al. (2002), Lai et al. (2002), Obata et al. (2006), and Wilson-Gerwing et al. (2009) to decrease target protein levels in the sensory system *in vivo*. The infusion of p75NTR AS was demonstrated to effectively downregulate p75NTR protein levels in intact sensory neurons by Obata et al. (2006) compared to the infusion of the SC sequence or a vehicle infusion, however no significant difference was observed compared to non-infused controls. Before utilizing this technology, it is therefore necessary to verify the effectiveness of the AS technology and to include controls for the intrathecal infusion technique (See Figure 1.5).

1.6 Rationale

Cellular signals underlying the development of neuropathic pain states in sensory ganglia following peripheral nerve injury are complex and include both positive signals generated as a result of the injury and negative signals due to altered availability to trophic support as a result of the injury. While it is well accepted that injury-associated alterations in the phenotype and properties of the injured neuron contribute to this state,

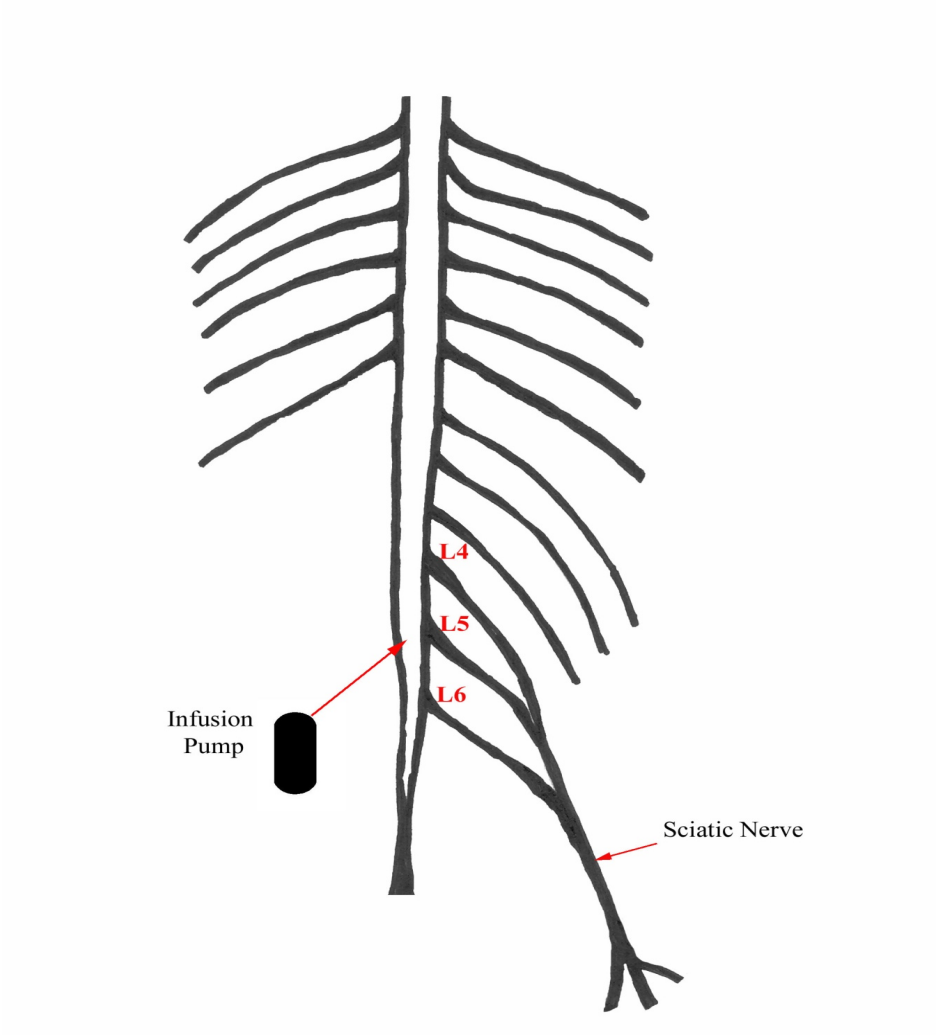

Figure 1.5 Intrathecal infusion technique. Silastic tubing attached to an osmotic mini-pump is inserted under the dura at the lumbar sacral junction and is slid rostrally to the level of the L5 dorsal root ganglia (DRG). The AS is taken up presumably by the axons in the spinal and ventral roots to the neurons L4/L5/L6 DRG and the lumbar region of the spinal cord.

this is not the only axis impacted. It is now becoming evident that injury induces changes in perineuronal glia and neighboring uninjured neuron phenotype that also drive this neuropathic state.

Gaining a better understanding of the nature of the signals underlying these changes may help identify novel therapeutic targets to help attenuate pain states. As neurotrophins have been shown to be major modulators of the phenotypic and physiological properties of this state by signaling via two very different classes of signaling molecules, I sought to determine the extent to which altered neuronal signaling via the common neurotrophin receptor p75NTR might impact the properties of intact sensory neurons such that they adopt an injury phenotype akin to that observed in the neuropathic pain state associated with nerve injury. Intact neurons were examined so as to isolate the impact of attenuated p75NTR expression from the plethora of additional molecules altered as a result of the nerve injury.

2. Hypothesis and Specific Aims

Supported by the data presented in the introduction, I hypothesize that ***reduced neuronal p75NTR expression leads to a reduction in neurotrophin signaling and altered neuron/perineuronal glial communication that results in induction of an injury phenotype associated with a neuropathic pain state.***

2.1 Specific Aims

To test this hypothesis, I addressed the following objectives:

(i) Examine whether intrathecal infusion of p75NTR AS is selectively taken up by neurons and not nonneuronal cells in the DRG as was observed for intrathecally delivered siRNA (Geremia et al., 2010);

(ii) Examine the impact of reduced neuronal p75NTR on a phenotype associated with neuropathic pain states, namely $Na_V1.8$, $Na_V1.9$ and TrkA, bearing in mind that inducing a dramatic shift in neuronal phenotype markers in intact/uninjured neurons is difficult (Wilson-Gerwing et al., 2008);

(iii) As reduced p75NTR expression is normally only observed in sensory neurons following peripheral nerve axotomy, I postulate that reduced neuronal p75NTR will result in altered communication between neurons and perineuronal/glial cells that mimics axotomy, thereby the shifting the expression of perineuronal/glial phenotypic markers, p75NTR, GFAP, $K_{ir}4.1$, from that of an intact to a reactive state. Thus, I will examine the impact of reducing neuronal p75NTR expression on perineuronal expression of markers associated with the reactive state.

3. Materials and methods

All animal procedures were approved by the University of Saskatchewan's Committee on Animal Care and Supply under the guidelines of the Canadian Council on Animal Care (protocol 19920164). A total of 22 male Wistar rats (Charles River Laboratories, St.Constant, QC) weighing 250–300g were used for the following set of experiments with 17 undergoing pump implantation and the other five as procedural controls.

3.1 Pump implantation surgery

Animals were anesthesized with a gas mixture of 1.5-2.5% isoflurane/97.5-98.5% oxygen and given an analgesic, buprenorphine (Temgesic, sc, 0.1-0.2 mg/kg), pre-surgically and post-surgically. The rats underwent implantation of a mini-osmotic pump attached to sterile silastic tubing (Model 2001, Alza, Cupertino, CA), inserted at the lumbar sacral junction as per Verge et al. (1989) such that the tip of the silastic tubing lay intrathecally at the level of the L5 DRG.

3.1.1. Osmotic pump preparation

Under sterile conditions, mini-osmotic pumps (Model 2001, Alza, Cupertino, CA) were assembled. Each vial of oligonucleotide (OGN) purchased from either Genelink (NY, USA) or Biognostik (Gottingen, Germany) contained 100 μg. This was dissolved in 225 μl of provided PBS dilution buffer, containing rat serum albumin (1 mg/μl), streptomycin (100 U/ml) and penicillin (100 U/ml). This resulted in enough solution to

inject into one pump. The pumps were then attached to silastic tubing and placed in 0.1 M PBS solution overnight to prime.

3.1.2 Intrathecal delivery of p75 NTR anti-sense and sense control oligonucleotides

Pumps continuously infused one of either four OGNs: fluorescein-tagged p75NTR OGN AS (5' – CAGGGCGGCTAAAAG – 3' (Genelink, NY, USA) (p75NTR-F AS), fluorescein-tagged p75NTR OGN SC (5' – ACTACTACACTAGACTAC – 3') (Biognostik, Gottingen, Germany) (p75NTR-F SC), p75NTR OGN AS (5' – CAGGGCGGCTAAAAG – 3' (Biognostik, Gottingen, Germany) (p75NTR AS) or p75NTR OGN SC (5' – ACTACTACACTAGACTAC – 3') (Biognostik, Gottingen, Germany) (p75NTR SC). The fluorescein-tagged OGNs were infused in order to visualize their uptake pattern. The pumps infused the OGNs at a rate of 1 μl/hr and a concentration of 0.5 nmol/ml.

3.1.3 Animal perfusion and tissue preparation

The rats were killed at various time points according to the following criteria: p75NTR-F AS (n=2) 36 hours post-implantation surgery, p75NTR-F AS (n=2) 48 hours post-implantation surgery and p75NTR-F SC (n=3), p75NTR AS (n=5) and p75NTR SC (n=5) 7 days post-implantation surgery (control animals (n=5) were killed at the same time as the p75NTR AS and p75NTR SC groups). The rats were deeply anesthetized and perfused transcardially with 0.1 M PBS, pH 7.4, followed by 4% paraformaldehyde in 0.1 M PBS. The sciatic nerves, spinal cord, bilateral L4, L5 and L6 dorsal root ganglia were carefully removed and postfixed for 1-1.5h in 4% paraformaldehyde, then cryoprotected

in 10% sucrose followed by 20 % sucrose overnight at 4°C. All ganglia were frozen with OCT compound (Tissue Tek; Miles Laboratories, Elkhart, IN) in a cryomold (Tissue Tek, Miles Laboratories). The L4 and L5 ganglia were then cut transversally on a cryostat (Micron, Zeiss, Canada) into 8 μm sections and thaw mounted on Superfrost Plus slides (Fisher Scientific).

3.2 Immunohistochemistry

3.2.1 Visualization of fluorescein-tagged oligonucleotide

The slides were then washed three times in 0.1M PBS and coverslipped using Prolong Gold anti-fade reagent (Invitrogen – Molecular Probes #P36930, Carlsbad, CA). Images were taken on a Zeiss Axio Imager 1 system with Northern Eclipse® (Empix Imaging Inc.) software.

3.2.2 Visualization of protein expression with antibodies

One week post-implantation surgery, the rats were deeply anesthesized and perfused transcardially with 0.1 M PBS, pH 7.4, followed by 4% paraformaldehyde in 0.1 M PBS. The sciatic nerve, spinal cord, bilateral L4, L5 and L6 dorsal root ganglia were carefully removed and postfixed for 1-1.5h then cryoprotected in 10% sucrose followed by 20 % sucrose overnight at 4°C. Experimental and control ganglia were embedded in a cryomold (Tissue Tek; Miles Laboratories, Elkhart, IN) with OCT compound (Tissue Tek, Miles Laboratories) as to ensure parallel processing. The ganglia were then cut transversally on a cryostat (Micron, Zeiss, Canada) into 6 or 8 μm sections and thaw

mounted on Superfrost Plus slides (Fisher Scientific) for immunohistochemistry processing.

Slides were blocked with 5% normal goat serum, 5% normal horse serum, 1.5% bovine serum albumin and 0.3% Triton X-100 overnight in 0.1 M PBS and subsequently treated with the following primary antibodies: mouse anti-p75NTR (Santa Cruz, CA, USA, 1:500), rabbit anti-$Na_V1.8$ channel (Chemicon Intl, 1:200), rabbit anti-$Na_V1.9$ channel (Chemicon Intl, 1:100), rabbit anti-TrkA neurotrophin receptor (Cedarlane Laboratories, 1:1500), rabbit anti-GFAP (Dakopatts, 1:1000), mouse anti-connexin 43 (Millipore Inc., 1:100) or rabbit anti-$K_{ir}4.1$ channel (Alomone Inc., 1:500), in a solution comprised of 1% normal goat serum, 1% normal horse serum, 0.5% bovine serum albumin and 0.3% Triton X-100 overnight in 0.1 M PBS. After 0.1M PBS washes, the localization of the primary antibodies was visualized with goat anti-mouse Texas Red secondary antibody (Jackson Laboratories, USA, 1:200), goat anti-rabbit Alexa Fluor 488 (Invitrogen, 1:250) or donkey anti-rabbit Cy3 $F(ab')_2$ fragment (Jackson Laboratories Inc., 1:600) in a 0.1 M PBS solution at room temperature for one hour. Slides that were treated with anti-GFAP were subsequently double labeled with FITC-conjugated isolectin from *griffonia simplicifolia* (IB4) (Sigma, 1:20) in 0.1 M PBS at room temperature for 3 hours. The slides were then washed three times in 0.1M PBS and coverslipped using Prolong Gold anti-fade reagent with or without DAPI (Invitrogen – Molecular Probes #P36930, Carlsbad, CA). Control sections were processed in the same manner while omitting the primary antibody. All slides were stored at -20 degrees Celsius in light-proof bags.

3.3 Quantification of immunohistochemical signal

All slides were initially analyzed qualitatively and relative changes in immunohistochemistry labeling intensity between experimental groups were noted within each slide to omit discrepancies in background labeling intensity. Slides were selected for experimental analysis such that they had similar numbers of neurons in the DRG sections representing each experimental group. Analysis for p75NTR immunoreactive labeling intensity was performed on 9 DRG or 1208 neuronal profiles/1217-1568 SGC profiles (non-infused: n=3 animals; p75NTR SC: n=3 animals; p75NTR AS: n=3). Images were taken under identical conditions for each DRG on individual slides on a Zeiss Axio Imager 1 microscope system with Northern Eclipse® (Empix Imaging Inc.) software using a 40X objective for quantitative neuronal analysis and a 20x objective for sciatic nerve sections selected for demonstrating qualitative changes in immunohistochemistry labeling intensity. After photographing all sections on a slide, images of each DRG were taken with a 10X objective to create photomontages of each section to be analyzed which were then assembled using Adobe® Photoshop® CS3 (Adobe Systems Inc., USA) to ensure that all neurons with a visible nucleus were only traced once. For neuronal profiles, the circumference of the neuron was traced as well as the cytoplasm using tablet tracing software (Bamboo, Wacom Ltd, Japan) with inputs into Northern Eclipse® (Empix Imaging Inc.) which computed the diameter of each individual neuron and its respective average cytoplasmic immunohistochemical labeling intensity. For SGC profiles, the circumference of the neuron was traced as well as the cytoplasm of its SGCs representing average labeling intensity around that respective

neuron. Tablet tracing software (Bamboo, Wacom Ltd, Japan) was used with inputs into Northern Eclipse® (Empix Imaging Inc.) which computed the diameter of each individual neuron and the average immunohistochemical labeling intensity over the cytoplasm of the SGCs. Supplemental statistical and graphical manipulations were performed with Prizm 4.0 (Graph Pad Software, San Diego, CA) and Microsoft Excel (Microsoft Corp., USA).

3.4 Statistical analysis

Each data point for individual neurons analysed was normalized to the mean immunohistochemical labeling intensity from the non-infused control animal in its grouping using Microsoft Excel software (Microsoft Corp., USA). A Kruskall-Wallis non-parametric ANOVA (Prizm 4.0, Graph Pad Software, San Diego, CA) was used to compare the cytoplasmic immunohistochemistry labeling intensity between treatment groups. Statistical significance was accepted at $p < 0.05$ level. Supplemental statistical and graphical manipulations were performed with Prizm 4.0 (Graph Pad Software, San Diego, CA) and Microsoft Excel (Microsoft Corp., USA).

4. Results

4.1 Efficacy of intrathecal p75NTR anti-sense oligonucleotide treatment

Fluorescein-tagged p75NTR AS was infused intrathecally for 36 hours (n=2), 48 hours (n=2) or 7 days (n=3) to determine its localization following uptake. The fluorescence was observed in and around the spinal cord at all time points. The fluorescein signal was visualized in the DRG neurons as early as 36 hours, was more robust 48 hours after pump implantation (Figure 4.1.1), and was significantly reduced by 7 days (Data not shown). The fluorescein signal was not observed in the SGCs or in the extracellular space (Figure 4.1.1).

It should be noted that for all markers examined, similar qualitative trends were observed between experimental groups for all animals in each experimental group. Three grouping of different experimental animals were then selected for detailed quantitative analysis based on the observation that similar numbers of neurons were present in each of the DRG sections on the slide.

In order to confirm the efficacy of the intrathecally infused p75NTR AS, sections processed for immunohistochemistry were analyzed to detect changes in the immunoreactive labeling intensity of p75NTR. A 7 day intrathecal infusion of p75NTR AS resulted in significantly decreased neuronal cytoplasmic immunoreactive labeling intensity of p75NTR over all size ranges of neurons compared to non-infused and p75NTR SC controls in the DRG (Figures 4.1.2, 4.1.3 and 4.1.4). Each neuronal data point was normalized to the mean immunohistochemical labeling intensity from the non-

infused control animal in its grouping. It was thus observed that the percentage of data points above the mean immunohistochemical labeling intensity of the non-infused control

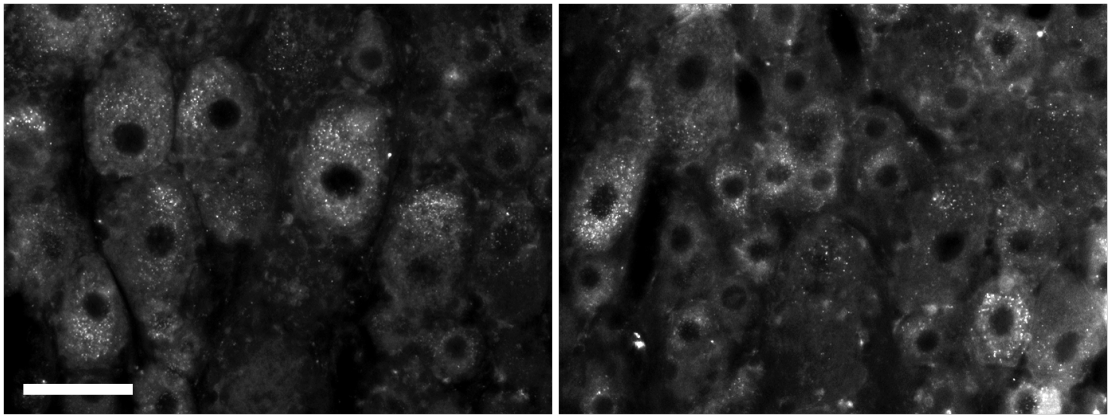

Figure 4.1.1 Intrathecally infused fluorescein-tagged p75NTR AS localizes to sensory neurons in the dorsal root ganglion and not surrounding satellite glial cells. Fluorescence photomicrographs of L5 DRG sections processed for immunohistochemistry depict the localization of fluorescein-tagged p75NTR AS OGN in small, medium and large neurons however not in the SGCs in L5 DRG from uninjured intact rats. Scale bar = 50 μm

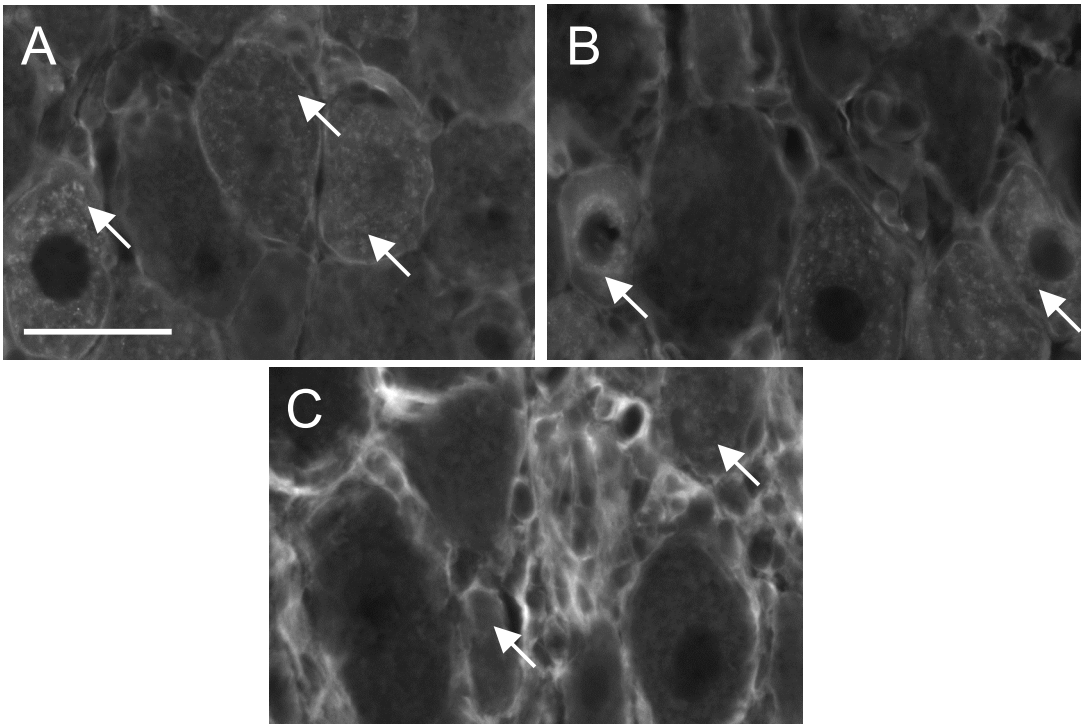

Figure 4.1.2 Cytoplasmic neuronal p75NTR immunoreactive labeling intensity is decreased with p75NTR AS. Fluorescence photomicrographs of L5 DRG sections processed for immunohistochemistry depict p75NTR protein expression in the cytoplasm of neurons in L5 DRG from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) results in decreased immunoreactive labeling intensity of p75NTR protein in neurons compared to control non-infused (A) and p75NTR SC OGN infused (B) treatment groups. This decreased immunoreactive labeling intensity of p75NTR is most evident in the heavily labeled population (Arrows). Infusion of p75NTR AS does however result in increased immunoreactive labeling intensity of p75NTR protein in SGCs (discussed in Results Section 5.4.2). Scale bar = 50 μm

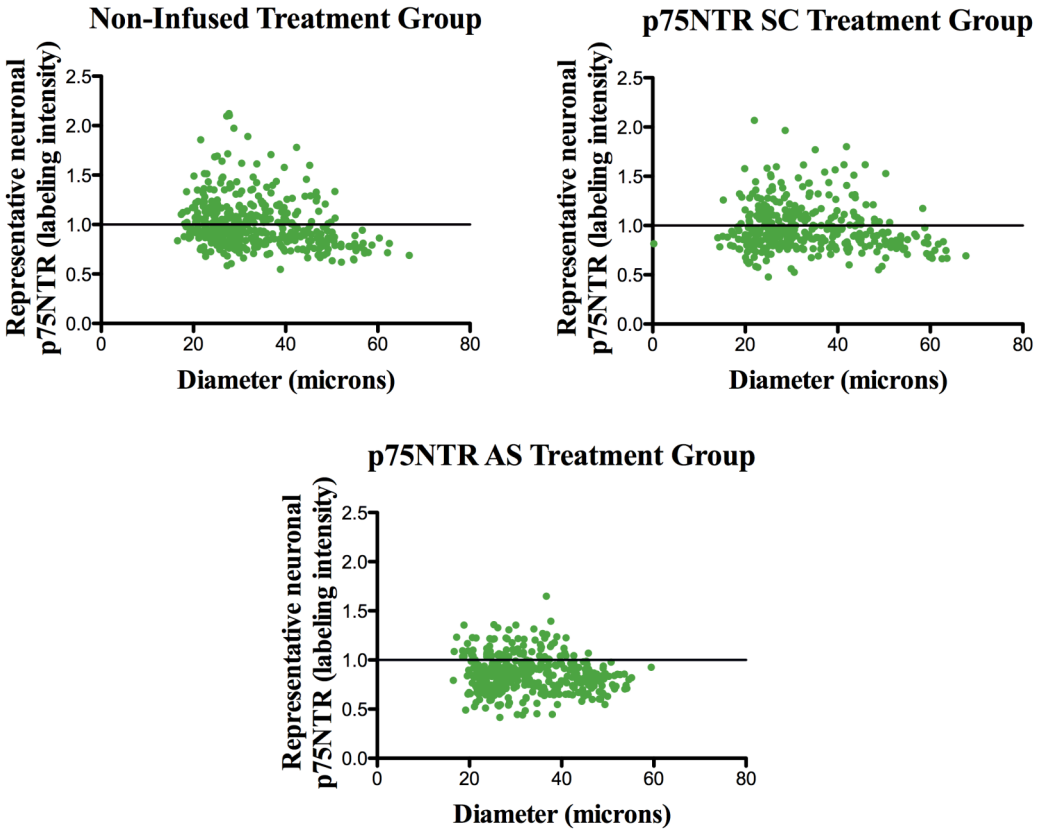

Figure 4.1.3 Cytoplasmic neuronal p75NTR immunoreactive labeling intensity is decreased with p75NTR AS. Scatter plots depict quantification of immunoreactive labeling intensity of p75NTR protein expression in the cytoplasm of neurons in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean labeling intensity in neurons from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) results in a decrease in expression of p75NTR in neurons compared to non-infused (n=3) and p75NTR SC OGN infused (n=3) treatment groups. N= A total of 375 to 445 neurons analyzed per treatment group.

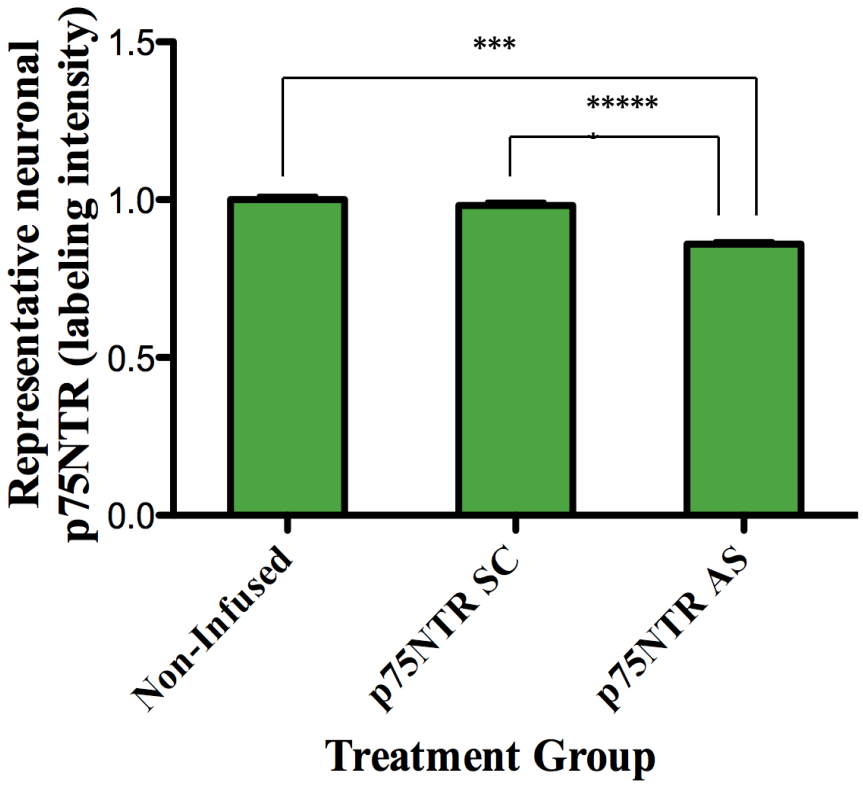

Figure 4.1.4 Mean cytoplasmic neuronal p75NTR immunoreactive labeling intensity is significantly decreased with p75NTR AS. Graph depicts quantification of relative changes in mean immunoreactive labeling intensity of p75NTR protein expression in the cytoplasm of neurons in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean labeling intensity in neurons from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) results in a significant decrease in expression of p75NTR in neurons compared to non-infused (n=3) and p75NTR SC OGN infused (n=3) treatment groups. Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; * $p<0.01$, *** $p<0.0001$). Bars represent the standard error of the mean (s.e.m.). N= A total of 375 to 445 neurons analyzed per treatment group.

group (>1.00) from the p75NTR AS infused group decreased to 20.36% (0.013133 s.e.m.) from 39.78% (0.017141 s.e.m.) for the non-infused control group and 36.80% (0.016958 s.e.m.) for the p75NTR SC infused group (Figure 4.1.3). The average mean immunoreactive labeling intensity of p75NTR was significantly lower for the p75NTR AS treated group compared to the p75NTR SC treated and the non-infused groups (0.8458 +/- 0.00940 (s.e.m.) compared to 0.9812 +/- 0.01214 (s.e.m.) and 1.0000 +/- 0.01154 (s.e.m.) respectively) (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.0001$) (Figure 4.1.4). It was noted however that the p75NTR AS infusion produced a marked increase in the immunoreactive labeling intensity of p75NTR in the SGCs (See Section 4.1.2).

4.2 Impact of reduced neuronal p75NTR expression on phenotype associated with neuropathic pain states in intact sensory neurons

4.2.1 Neuronal Na^+ channel expression

Immunohistochemistry revealed that in the intact state, independent of infusion treatment, $Na_V1.8$ protein was observed most prominently in the smaller DRG neurons, with some medium to large sized neurons also expressing low to moderate levels of $Na_V1.8$ protein (Figure 4.2.1.1). A 7 day intrathecal infusion of either p75NTR AS or p75NTR SC was ineffective at altering levels of neuronal $Na_V1.8$ protein compared to the non-infused treatment group. Each data point was normalized to the mean immunohistochemical labeling intensity from the non-infused control animal in its grouping. It was thus observed that the percentage of data points above the mean immunohistochemical labeling intensity of the non-infused control group (>1.00) from

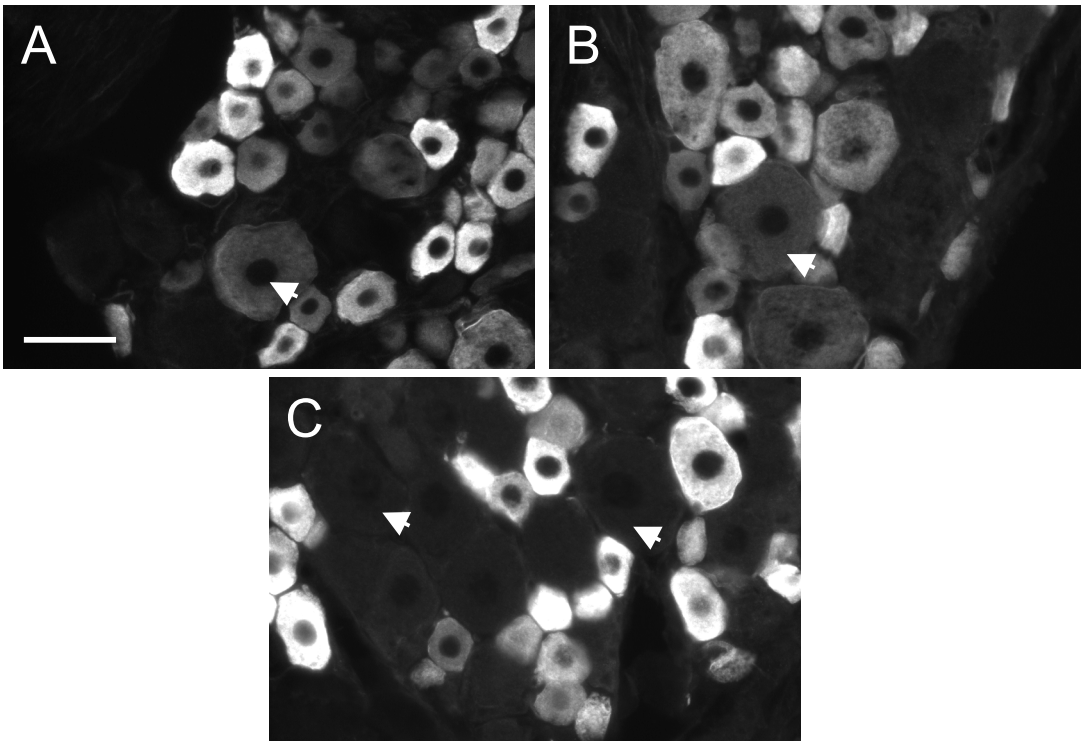

Figure 4.2.1.1 Neuronal $Na_V1.8$ immunoreactive labeling intensity in DRG does not discernably change with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Fluorescence photomicrographs of L5 DRG sections processed for immunohistochemistry depict $Na_V1.8$ protein expression in the cytoplasm of neurons in L5 DRG from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) or p75NTR SC OGN (B) does not result in discernable change in $Na_V1.8$ protein expression compared to non-infused (A) controls in small-medium neurons. However, there appears to be reduced expression in the very large neurons (Arrows). Scale bar: 50 μm

the p75NTR AS infused group did not vary markedly compared to p75NTR SC infused or the non-infused control group (38.88% (0.02332 s.e.m.) compared to 40.19% (0.02694 s.e.m.) and 45.57% (0.02931 s.e.m.) respectively) (Figure 4.2.1.2). This was also shown in terms of the mean immunoreactive labeling intensity of $Na_V1.8$ with no significant differences between non-infused (1.0000 +/- 0.02931 (s.e.m.)), p75NTR SC infused (0.9414 +/- 0.02694 (s.e.m.)) or the p75NTR AS infused (0.9005 +/- 0.02332 (s.e.m.)) treatment groups (Figure 4.2.1.3). We observed however a small decrease in the percentage of data points above 1.00 in the larger sized neurons (diameter >50 mm) of the p75NTR AS treated group (0.362% (0.02332 s.e.m.) compared to the p75NTR SC treated (3.962% (0.02694 s.e.m.) and the non-infused control group (6.048% (0.0293 s.e.m.)) (Figure 4.2.1.2). This was also shown in terms of the mean immunoreactive labeling intensity of $Na_V1.8$ being slightly decreased in larger sized neurons (diameter >50 mm) following p75NTR AS infusion (0.3737 +/- 0.02700 (s.e.m.) compared to no infusion (0.6269 +/- 0.05247 (s.e.m.)) (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.01$) (Figure 4.2.1.3).

The levels of observed immunoreactive labeling intensity of $Na_V1.8$ in sections of DRG were consistent with those observed in their corresponding intact sciatic nerve (Figure 4.2.1.4). This suggests that lack of variability in the observable $Na_V1.8$ protein levels between experimental groups was not due to shuttling of the protein to the axons from the cell bodies.

Immunohistochemistry revealed that in the intact state, independent of infusion treatment, $Na_V1.9$ protein was observed most prominently in the smaller DRG neurons, with some medium to large sized neurons also expressing low to moderate levels of

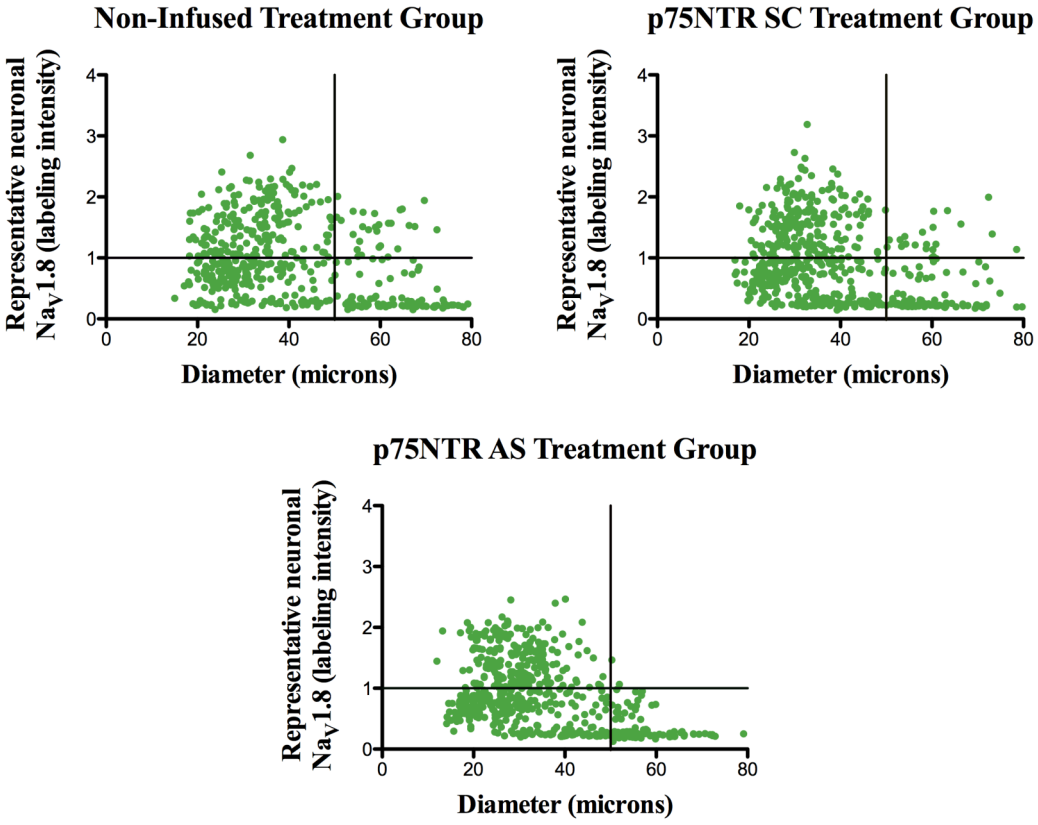

Figure 4.2.1.2 Neuronal $Na_V1.8$ immunoreactive labeling intensity in DRG does not vary with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Scatter plots depict quantification of immunoreactive labeling intensity of $Na_V1.8$ protein expression in neurons in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean signal labeling intensity in neurons from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) or p75NTR SC OGN (n=3) did not result in any noted changes in the expression levels of $Na_V1.8$ protein in small-medium neurons compared to the non-infused (n=3) treatment group. However, there is a decrease in expression in the larger (>50 μm) neurons. N= A total of 463 to 552 neurons analyzed per treatment group.

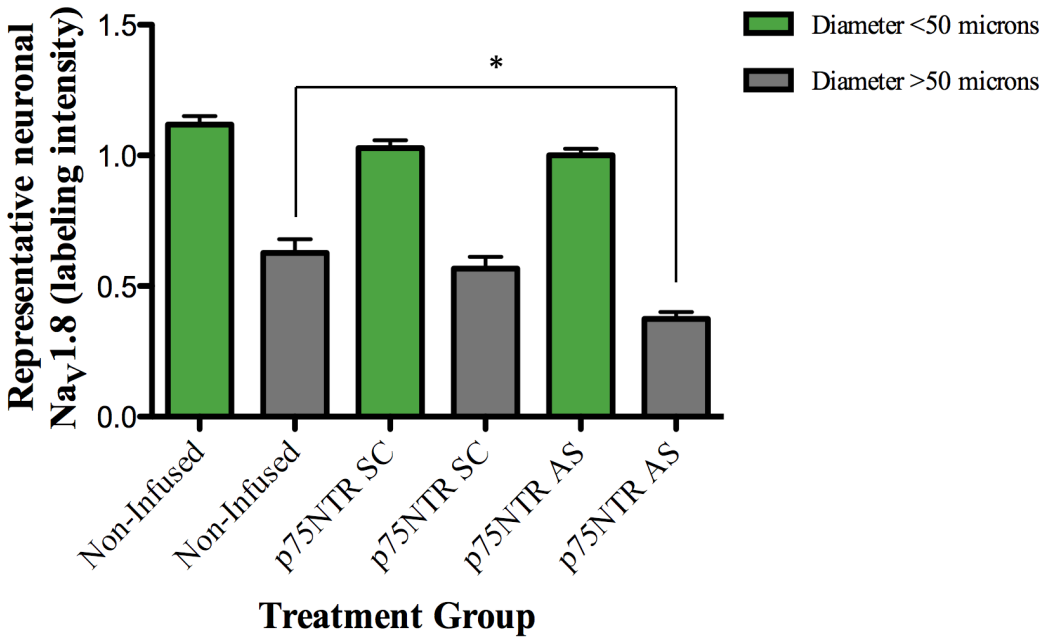

Figure 4.2.1.3 Mean neuronal $Na_V1.8$ immunoreactive labeling intensity in DRG does not vary greatly with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Graph depicts quantification of immunoreactive labeling intensity of $Na_V1.8$ protein expression in neurons in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean signal labeling intensity in neurons from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) or p75NTR SC OGN (n=3) did not result in any significant changes in the expression levels of $Na_V1.8$ protein in small-medium neurons compared to the non-infused (n=3) treatment group. However, there was a slight decrease in $Na_V1.8$ protein expression in large neurons in the p75NTR AS OGN treated group compared to large neurons in the non-infused treatment group. Kruskal-Wallis test with Dunn's Multiple Comparison test; * $p<0.01$). Bars represent the standard error of the mean (s.e.m.). N= A total of 463 to 552 neurons analyzed per treatment group.

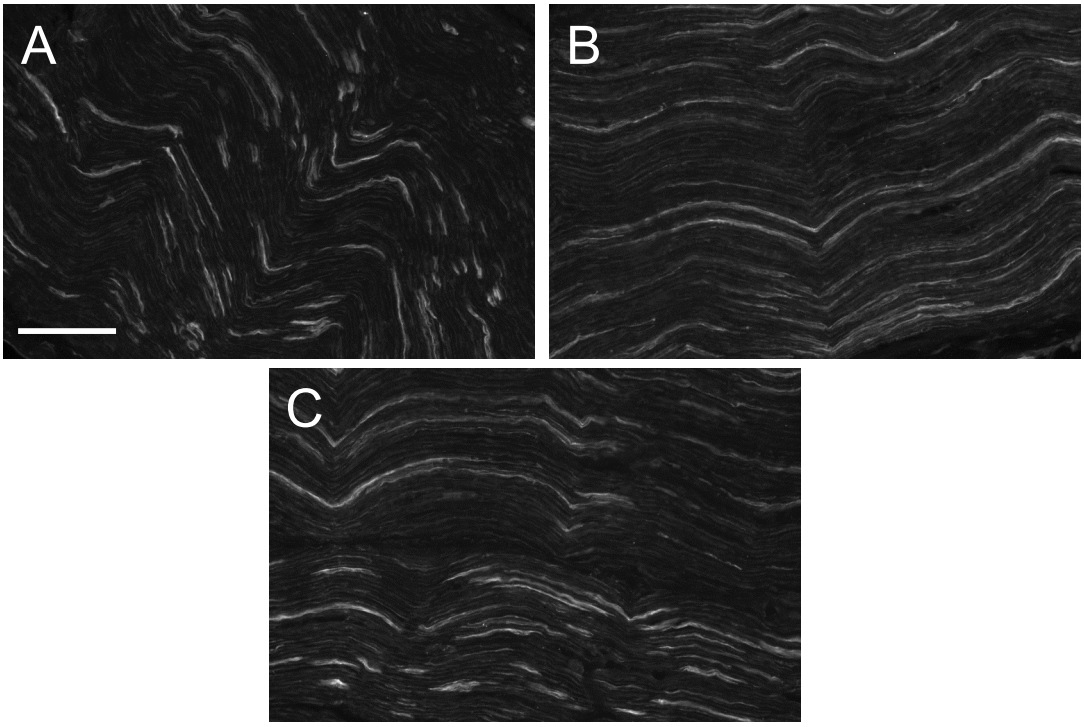

Figure 4.2.1.4 Neuronal $Na_V1.8$ immunoreactive labeling intensity in sciatic nerves does not discernably change with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Fluorescence photomicrographs of sciatic nerve sections processed for immunohistochemistry depict $Na_V1.8$ protein expression in axons from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) or p75NTR SC OGN (B) does not result in discernable changes in $Na_V1.8$ protein expression compared to non-infused (A) controls. Scale bar = 100 μm

$Na_V1.9$ protein (Figure 4.2.1.5). A 7 day intrathecal infusion of either p75NTR AS or p75NTR SC was ineffective at altering levels of neuronal $Na_V1.9$ protein compared to the non-infused treatment group. Each data point was normalized to the mean immunohistochemical labeling intensity from the non-infused control animal in its grouping. It was thus observed that the percentage of data points above the mean immunohistochemical labeling intensity of the non-infused control group (>1.00) from the p75NTR AS infused group did not vary markedly compared to p75NTR SC infused or the non-infused control group (40.85% (0.01527 s.e.m.) compared to 37.80%, 0.9330 (0.02002 s.e.m.) and 44.49% (0.01928 s.e.m.) respectively) (Figures 4.2.1.6). This was also shown in terms of the mean immunoreactive labeling intensity of $Na_V1.9$ with no significant differences between non-infused (1.0000 +/- 0.01928 (s.e.m.)), p75NTR SC infused (0.9330 +/- 0.02002 (s.e.m.)) or the p75NTR AS infused (0.9446 +/- 0.01527 (s.e.m.)) treatment groups (Figure 4.2.1.7).

The levels of expression observed in sections of DRG were consistent with those observed in their corresponding intact sciatic nerve (Figure 4.2.1.8). This suggests that lack of variability in the observable $Na_V1.9$ protein levels between experimental groups was not due to shuttling of the protein to the axons from the cell bodies.

4.2.2 Neuronal TrkA expression

Immunohistochemistry revealed that in the intact state, independent of infusion treatment, TrkA protein was observed most prominently in the smaller DRG neurons, with some medium to large sized neurons also expressing low to moderate levels of TrkA

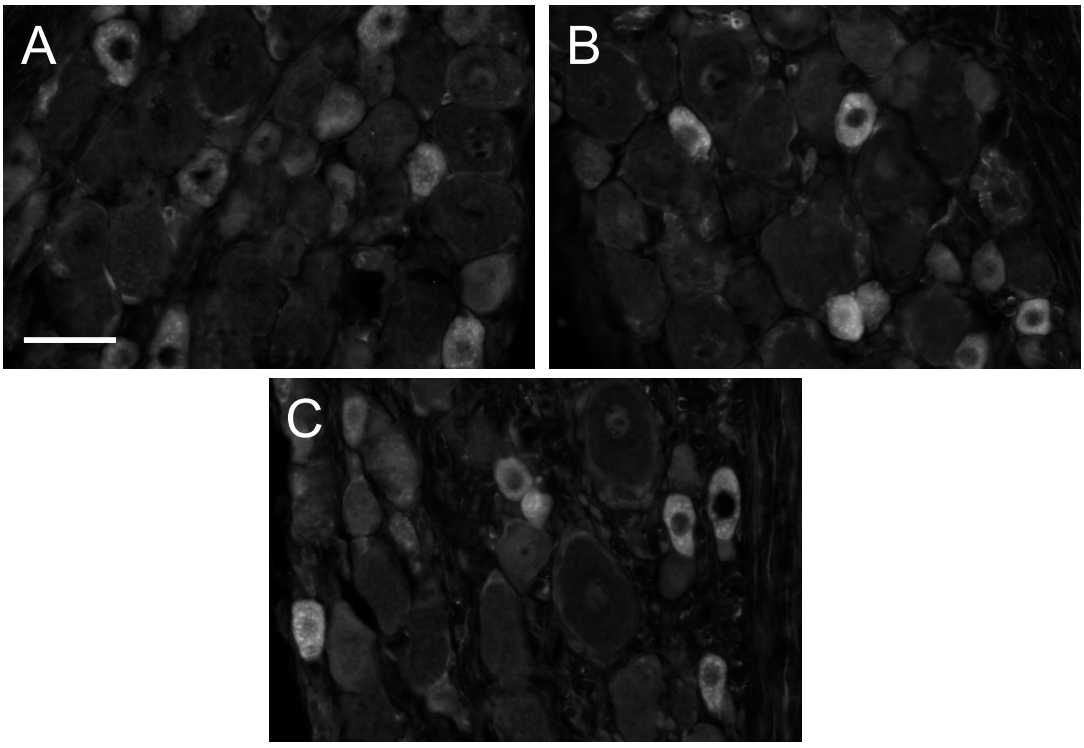

Figure 4.2.1.5 Neuronal $Na_V1.9$ immunoreactive labeling intensity in DRG does not discernably change with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Fluorescence photomicrographs of L5 DRG sections processed for immunohistochemistry depict $Na_V1.9$ protein expression in the cytoplasm of neurons in L5 DRG from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) or p75NTR SC OGN (B) does not result in any discernable change in $Na_V1.9$ protein expression compared to non-infused (A) controls. Scale bar: 50 μm

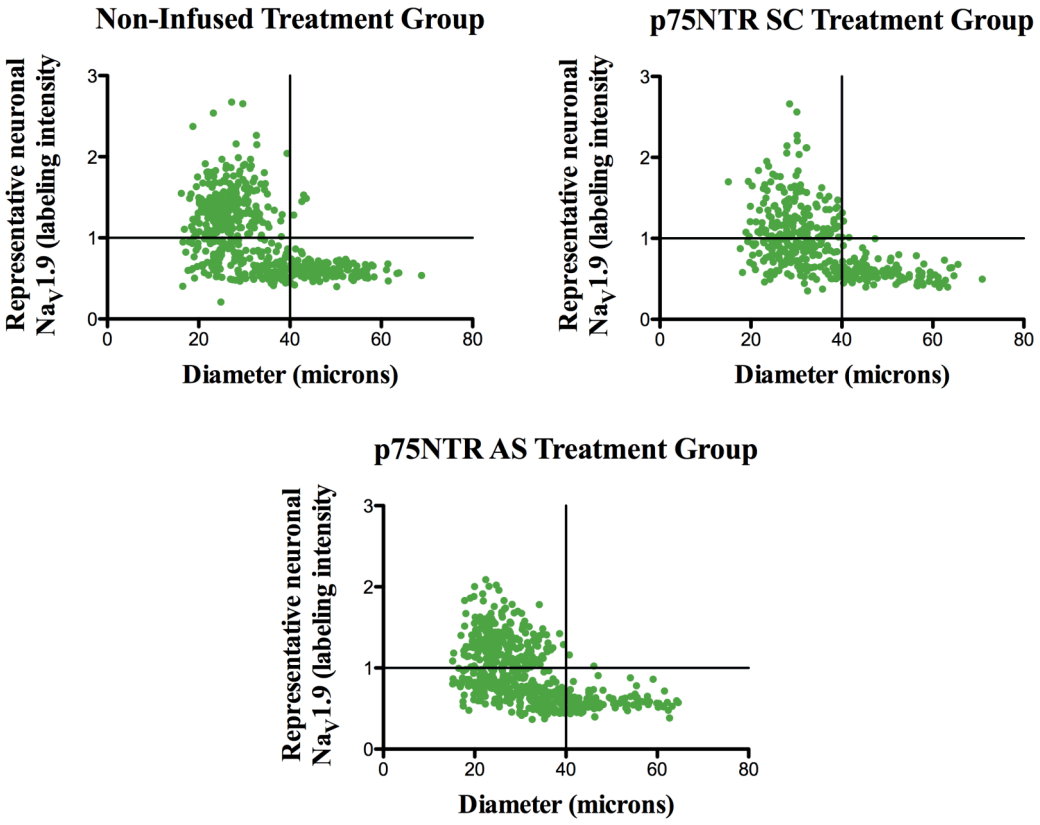

Figure 4.2.1.6 Neuronal $Na_V1.9$ immunoreactive labeling intensity in DRG does not vary with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Scatter plots depict quantification of immunoreactive labeling intensity of $Na_V1.9$ protein expression in neurons in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean signal labeling intensity in neurons from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) or p75NTR SC OGN (n=3) did not result in any noted changes in the expression levels of $Na_V1.9$ protein in neurons compared to the non-infused (n=3) treatment group. N= A total of 410 to 590 neurons analyzed per treatment group.

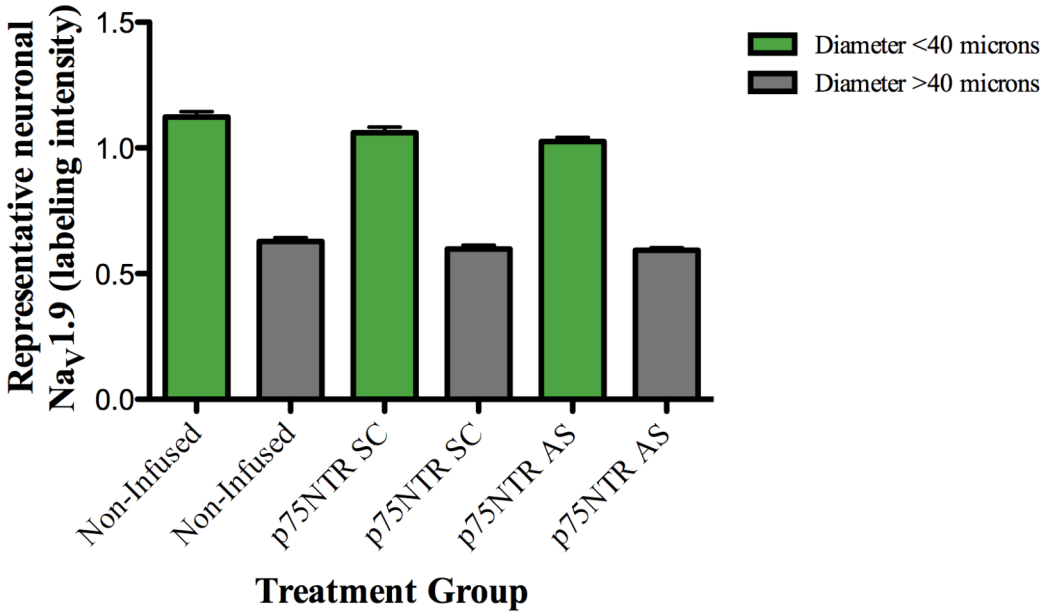

Figure 4.2.1.7 Mean neuronal $Na_V1.9$ immunoreactive labeling intensity in DRG does not vary significantly with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Graph depicts quantification of relative changes in mean immunoreactive labeling intensity of $Na_V1.9$ protein expression in neurons in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean signal labeling intensity in neurons from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) or p75NTR SC OGN (n=3) did not result in any significant differences in the expression levels of $Na_V1.9$ protein in neurons compared to the non-infused (n=3) treatment group. Bars represent the standard error of the mean (s.e.m.). N= A total of 410 to 590 neurons analyzed per treatment group.

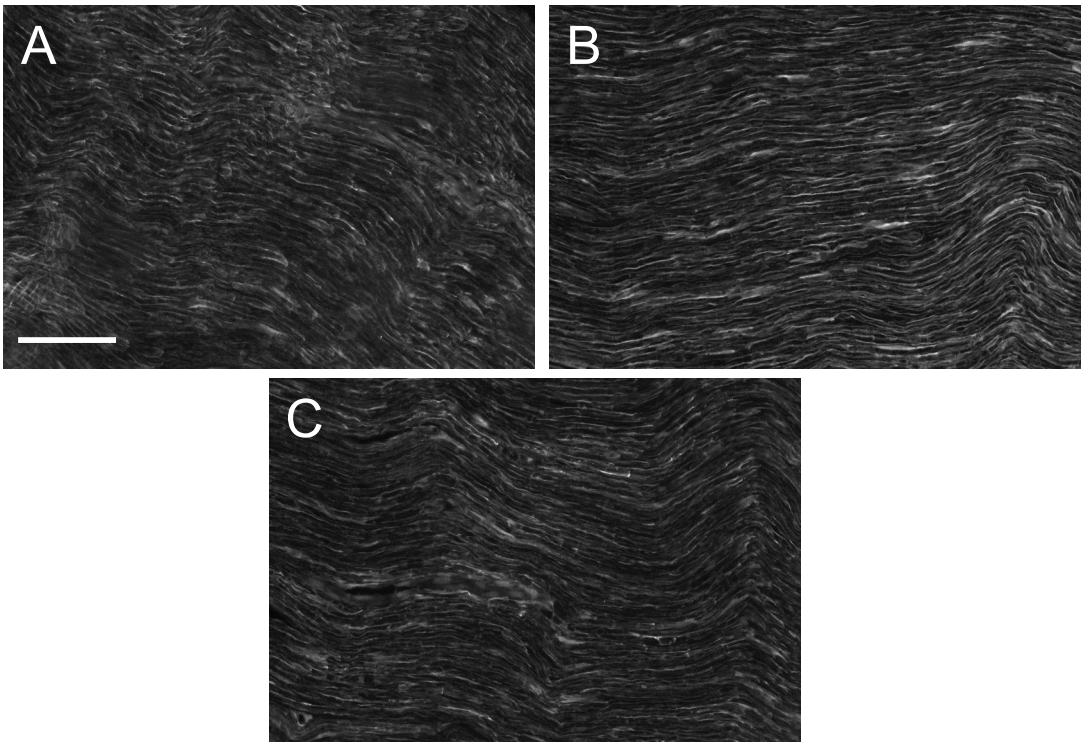

Figure 4.2.1.8 Neuronal $Na_V1.9$ immunoreactive labeling intensity in sciatic nerves does not discernably change with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Fluorescence photomicrographs of sciatic nerve sections processed for immunohistochemistry depict $Na_V1.9$ protein expression in the axons from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) or p75NTR SC OGN (B) does not result in any discernable change in $Na_V1.9$ protein expression compared to non-infused (A) controls. Scale bar: 100 μm

protein (Figure 4.2.2.1). A 7 day intrathecal infusion of either p75NTR AS or p75NTR SC was ineffective at altering levels of neuronal TrkA protein compared to the non-infused treatment group. Each data point was normalized to the mean immunohistochemical labeling intensity from the non-infused control animal in its grouping. It was thus observed that the percentage of data points above the mean immunohistochemical labeling intensity of the non-infused control group (>1.00) from the p75NTR AS infused group did not vary markedly compared to p75NTR SC infused or the non-infused control group (39.94% (0.01071 s.e.m.) compared to 35.33% (0.01474 s.e.m.) and 39.78% (0.01170 s.e.m.) respectively) (Figure 4.2.2.2). This was also shown in terms of the mean immunoreactive labeling intensity of TrkA with no significant differences between non-infused (1.0000 +/- 0.01170 (s.e.m.)), p75NTR SC infused (1.0144 +/- 0.01474 (s.e.m.) or the p75NTR AS infused (1.0078 +/- 0.01071 (s.e.m.) treatment groups (Figure 4.2.2.3).

The levels of expression observed in sections of DRG were consistent with those observed in their corresponding intact sciatic nerve (Figure 4.2.2.4). This suggests that lack of variability in the observable TrkA protein levels between experimental groups was not due to shuttling of the protein to the axons from the cell bodies. It is of note that some TrkA immunoreactivity was observed in the perineuronal area in all treatment groups with a slight increase seen with p75NTR AS treatment.

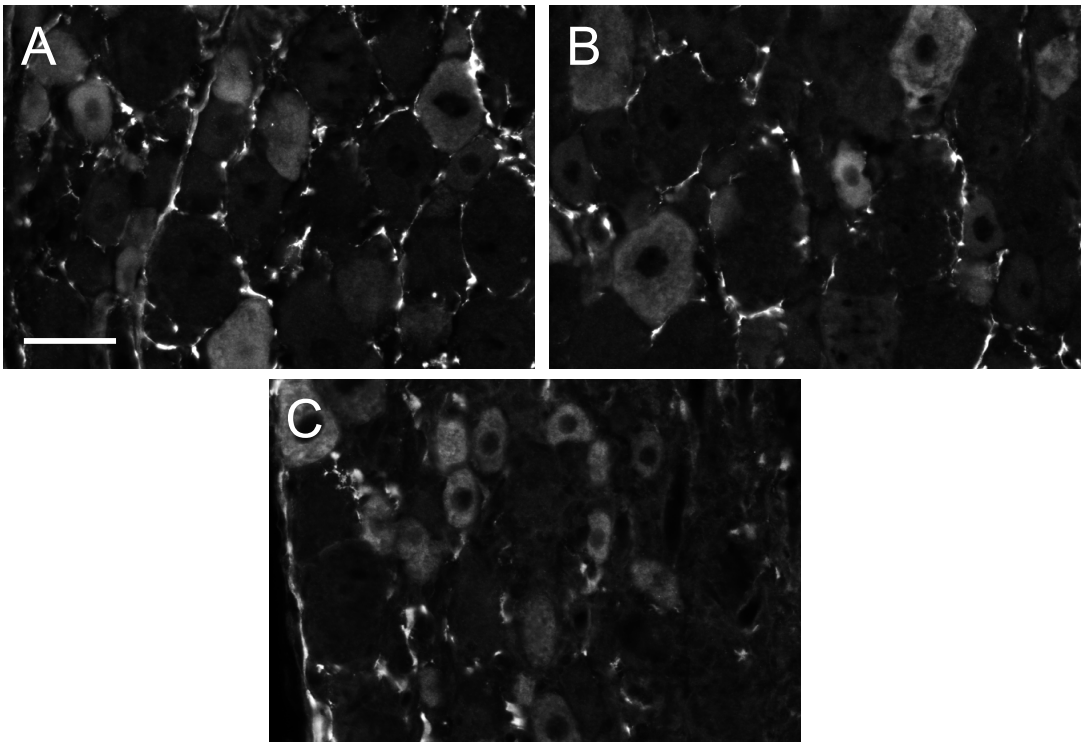

Figure 4.2.2.1 Neuronal TrkA immunoreactive labeling intensity in DRG does not discernably change with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Fluorescence photomicrographs of L5 DRG sections processed for immunohistochemistry depict TrkA protein expression in the cytoplasm of neurons in L5 DRG from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) or p75NTR SC OSN (B) does not result in any discernable change in TrkA protein expression compared to non-infused (A) controls. Scale bar: 50 μm

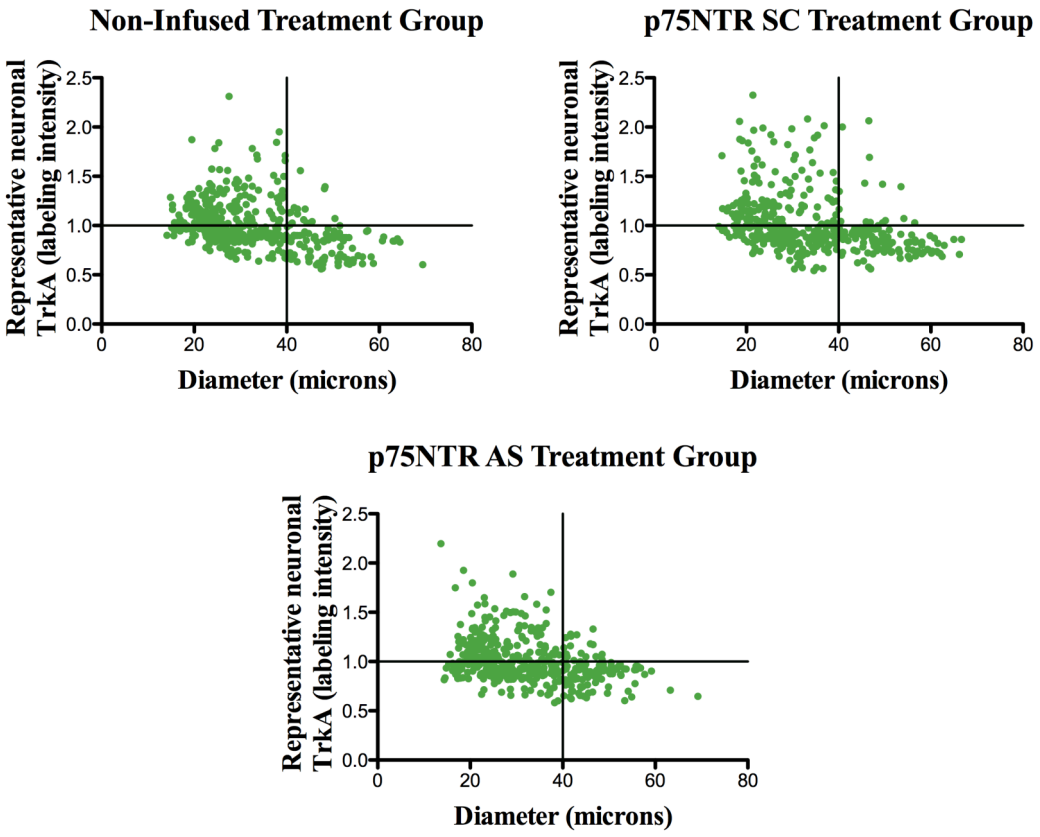

Figure 4.2.2.2 Neuronal TrkA immunoreactive labeling intensity in DRG does not vary with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Scatter plots depict quantification of immunoreactive labeling intensity of TrkA protein expression in neurons in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean signal labeling intensity in neurons from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) or p75NTR SC OGN (n=3) did not result in any noted changes in the expression levels of TrkA protein in neurons compared to the non-infused (n=3) treatment group. N= A total of 433 to 445 neurons analyzed per treatment group.

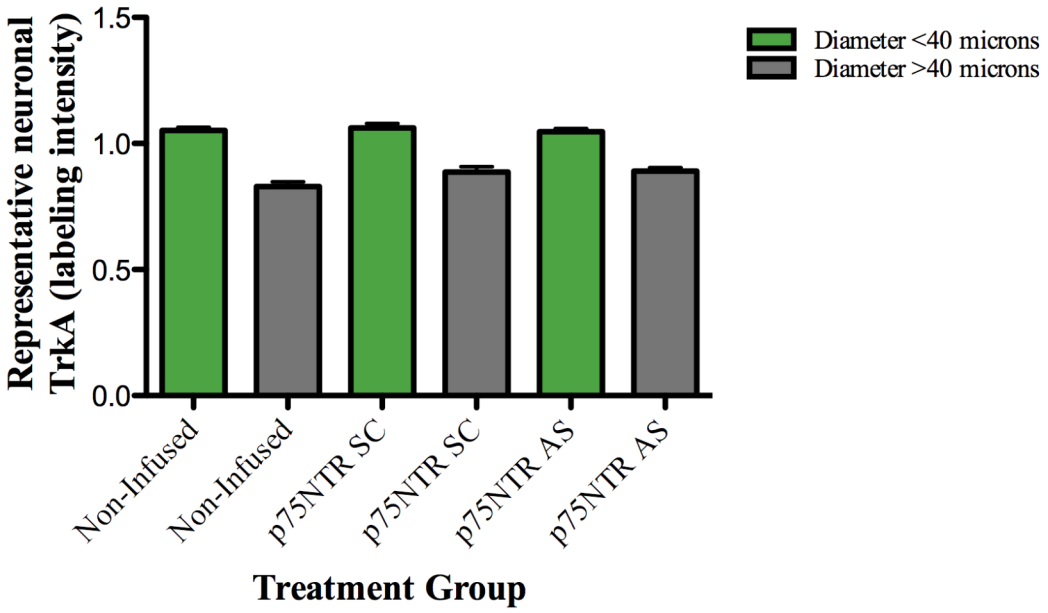

Figure 4.2.2.3 Mean neuronal TrkA immunoreactive labeling intensity in DRG does not vary significantly with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Graph depicts quantification of relative changes in mean fluorescence labeling intensity of TrkA protein expression in neurons in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean signal labeling intensity in neurons from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) or p75NTR SC OGN (n=3) did not result in any significant differences in the expression levels of TrkA protein in neurons compared to the non-infused (n=3) treatment group. Bars represent the standard error of the mean (s.e.m.). N= A total of 433 to 445 neurons analyzed per treatment group.

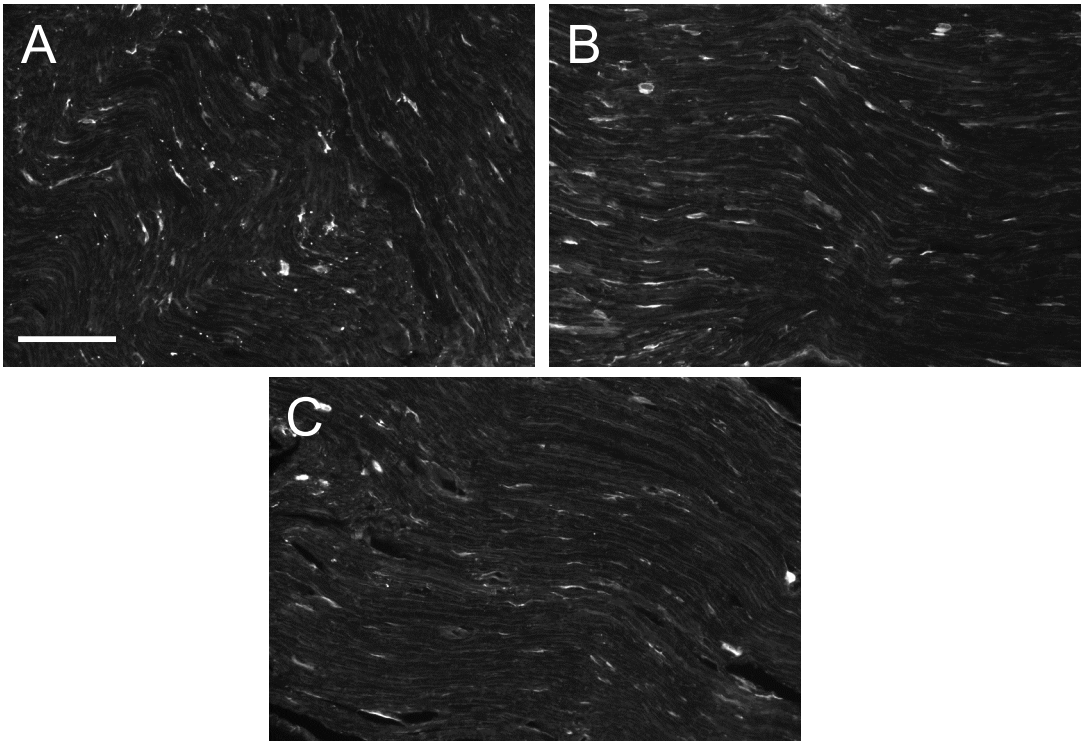

Figure 4.2.2.4 Neuronal TrkA immunoreactive labeling intensity in sciatic nerves does not discernably change with intrathecal infusion of either p75NTR AS OGN or p75NTR SC OGN compared to non-infused controls. Fluorescence photomicrographs of right sciatic nerve sections processed for immunohistochemistry depict TrkA protein expression in axons from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) or p75NTR SC OGN (B) does not result in any discernable change in TrkA protein expression compared to non-infused (A) controls. Scale bar: 100 μm

4.3 Impact of reduced neuronal p75NTR expression on satellite glial cell phenotype associated with neuropathic pain states

4.3.1 Satellite glial cell GFAP expression

Analysis of DRG sections processed for immunohistochemistry to detect glial levels of immunoreactive labeling intensity of GFAP protein revealed that 7 day intrathecal infusion of p75NTR AS resulted in increased the immunoreactive labeling intensity compared to non-infused and p75NTR SC controls (Figure 4.3.1.1). This increased expression of glial GFAP protein was also observed in sciatic nerve sections (Figure 4.3.1.2). Each data point was normalized to the mean immunohistochemical labeling intensity from the non-infused IB4+ control animal in its grouping. Neurons that did not label with isolectin from *griffonia simplicifolia* (IB4) had a higher immunoreactive labeling intensity (<2) of GFAP protein in their surrounding SGCs following p75NTR AS treatment (54.43% +/- 0.05400 (s.e.m.)) compared to p75NTR SC infusion (6.46% +/- 0.03390 (s.e.m.)) or non-infused controls (10.15%+/- 0.34223 (s.e.m.)) (Figure 4.3.1.3). Neurons that were negative for IB4 had a significantly higher mean immunoreactive labeling intensity of GFAP protein in their surrounding SGCs compared to neurons that did label for IB4 across all treatment groups (from 1.0000 +/- 0.02938 (s.e.m.) to 1.2681 +/- 0.03422 (s.e.m.) (non-infused), 0.8951 +/- 0.03098 (s.e.m.) to 1.1889 +/- 0.03390 (s.e.m.) (SC), 1.3745 +/- 0.03694 (s.e.m.) to 2.3273 +/- 0.05400 (s.e.m.) AS) (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.0001$) (Figure 4.3.1.4). The mean immunoreactive labeling intensity of GFAP in SGCs surrounding IB4-positive neurons increased significantly in the p75NTR AS infused (1.3745 +/- 0.03694 (s.e.m.)) treatment group compared to the non-infused (1.0000 +/-

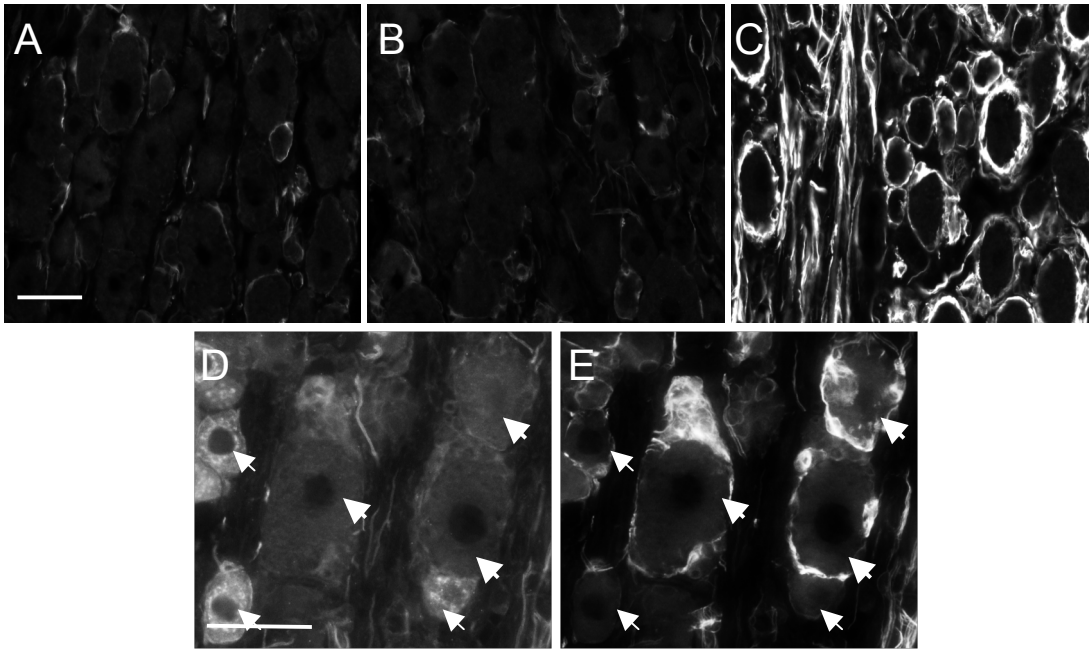

Figure 4.3.1.1 Satellite glial cell GFAP immunoreactive labeling intensity is dramatically increased in response to p75NTR AS treatment and is most evident in satellite glial cells surrounding the subpopulation of IB4 non-labeled neurons. Top: Fluorescence photomicrographs of L5 DRG sections processed for immunohistochemistry depict GFAP protein expression in the cytoplasm of SGCs in L5 DRG from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) results in increased expression of GFAP in SGCs compared to control non-infused (A) and p75NTR SC OGN infused (B) treatment groups. **Bottom:** Fluorescence photomicrographs of DRG sections that are processed for GFAP immunohistochemistry and double-labeled with FITC-conjugated lectin from *Griffonia simplicifolia* (IB4) in order to discern alterations in GFAP expression in SGCs (D) surrounding IB4-negative (large arrows) and IB4-positive neurons (small arrows) (E). Note: Expression of GFAP protein is higher in SGCs surrounding IB4-negative neurons compared to SGCs surrounding IB4-positive neurons. Scale bars = 50 μm

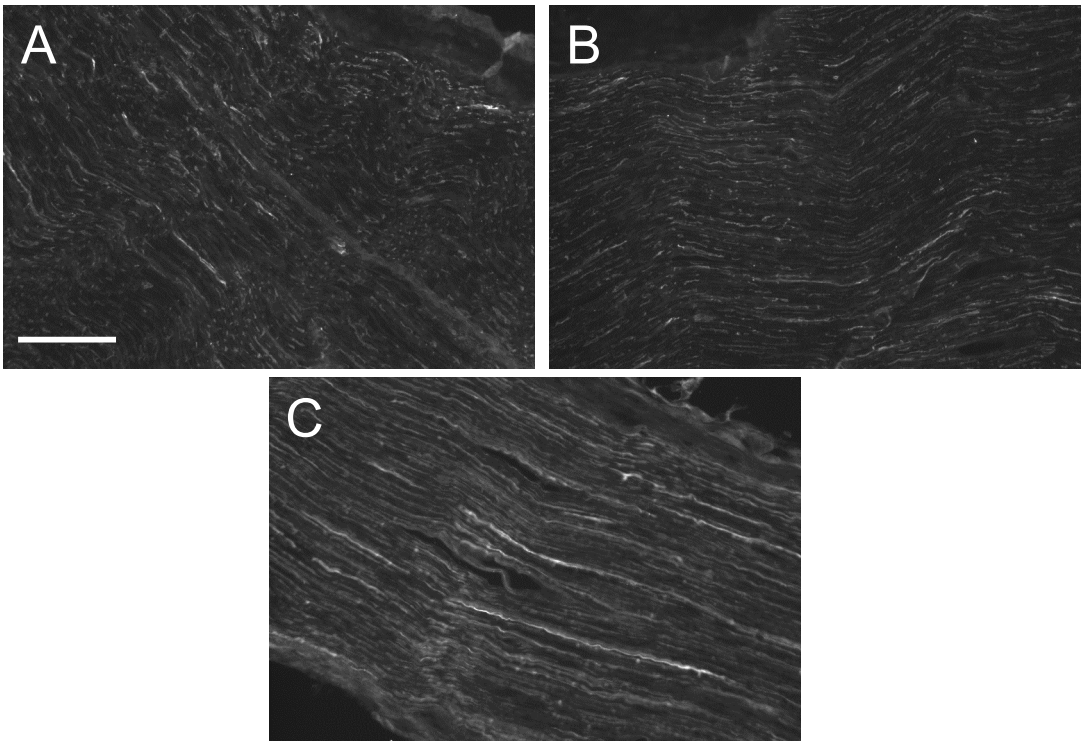

Figure 4.3.1.2 Glial/Schwann cells GFAP immunoreactive labeling intensity is dramatically increased in response to p75NTR AS treatment in sciatic nerves.
Fluorescence photomicrographs of sciatic nerve sections processed for immunohistochemistry depict GFAP protein expression in axons from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) results in increased expression of GFAP in glial/Schwann cells compared to control non-infused (A) and p75NTR SC OGN infused (B) treatment groups. Scale bar: 50 μm

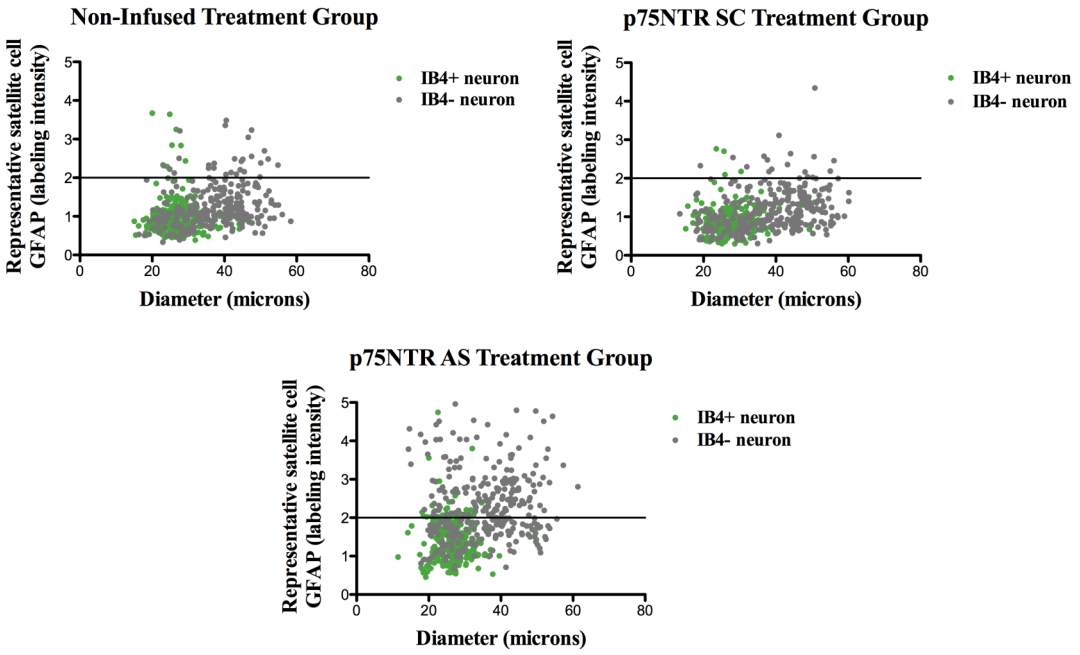

Figure 4.3.1.3 Satellite glial cell GFAP immunoreactive labeling intensity is increased with p75NTR AS treatment surrounding both IB4 positively labeled and IB4 non-labeled neurons. Scatter plots depict quantification of relative changes in normalized immunoreactive labeling intensity of GFAP protein expression in SGCs in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean signal labeling intensity in SGCs from the non-infused IB4+ control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) results in a significant increase in expression of GFAP in SGCs compared to non-infused (n=3) and p75NTR SC OGN infused (n=3) treatment groups. Within this population, SGCs surrounding IB4 negative neurons had a significantly higher expression of GFAP compared to IB4 positive neurons. N= A total of 515 to 538 SGCs analyzed per treatment group with a total of 3 animals in each group.

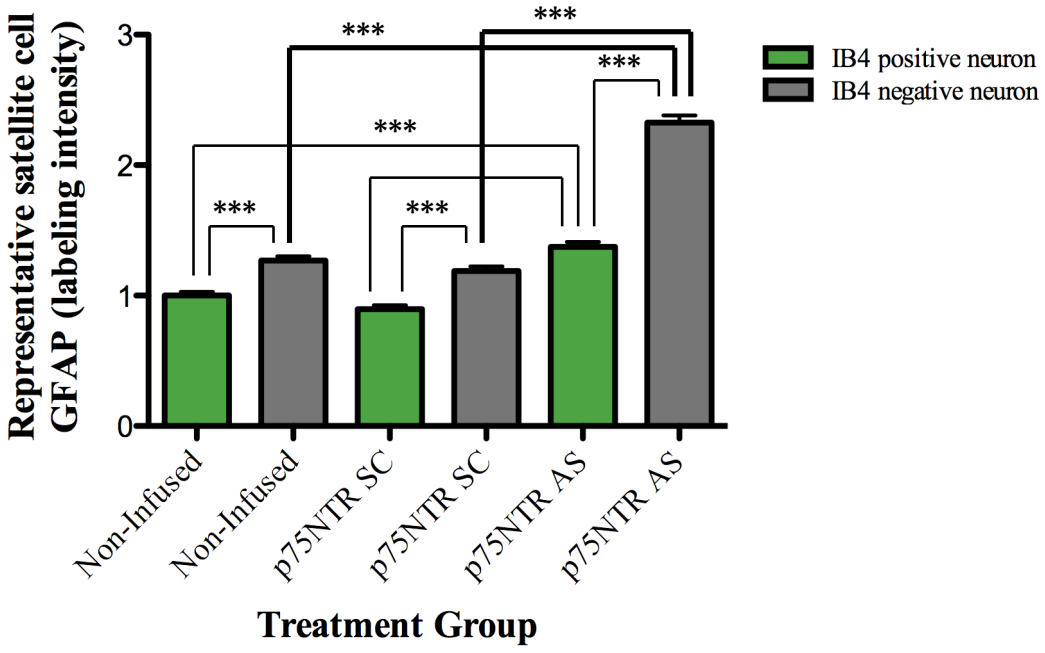

Figure 4.3.1.4 Mean satellite glial cell GFAP immunoreactive labeling intensity is significantly increased with p75NTR AS treatment surrounding both IB4 positively labeled and IB4 non-labeled neurons. Graph depicts quantification of relative changes in normalized mean immunoreactive labeling intensity of GFAP protein expression in SGCs in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean signal labeling intensity in SGCs from the non-infused IB4+ control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) results in a significant increase in expression of GFAP in SGCs compared to non-infused (n=3) and p75NTR SC OGN infused (n=3) treatment groups. Within this population, SGCs surrounding IB4 negative neurons had a significantly higher expression of GFAP compared to IB4 positive neurons. Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$). Bars represent the standard error of the mean (s.e.m.). N= A total of 515 to 538 SGCs analyzed per treatment group with a total of 3 animals in each group.

0.02938 (s.e.m.)) and p75NTR SC infused (0.8951 +/- 0.03098 (s.e.m.)) treatment groups. The mean immunoreactive labeling intensity of GFAP in SGCs surrounding IB4-negative neurons increased significantly in the p75NTR AS infused (2.3273 +/- 0.05400 (s.e.m.)) treatment group compared to the non-infused (1.2681 +/- 0.03422 (s.e.m.)) and p75NTR SC (1.1889 +/- 0.03390 (s.e.m.)) infused treatment groups.

The levels of observed immunoreactive labeling intensity of GFAP in sections of sciatic nerve were consistent with those observed in their corresponding intact DRG (Figure 4.3.1.2).

4.3.2 Satellite glial cell p75NTR expression

Analysis of sections processed for immunohistochemistry to detect glial expression of p75NTR protein revealed that a 7 day intrathecal infusion of p75NTR AS increased the immunoreactive labeling intensity compared to non-infused and p75NTR SC controls (Figure 4.3.2.1). Each data point was normalized to the mean immunohistochemical labeling intensity from the non-infused control animal in its grouping. It was thus observed that the percentage of data points above the mean immunohistochemical labeling intensity of the non-infused control group (>1.00) from the p75NTR AS infused group increased to 93.51% (0.02038 s.e.m.) from 45.67% (0.00898 s.e.m.) for the non-infused control group and 48.89% (0.01523 s.e.m.) for the p75NTR SC infused group (Figure 4.3.2.2). The average mean immunoreactive labeling intensity of p75NTR was significantly higher for the p75NTR AS treated group compared to the p75NTR SC treated and the non-infused groups (1.4935 +/- 0.02038 (s.e.m.) compared to 1.0168 +/- 0.01523 (s.e.m.) and 1.0000 +/- 0.00898 (s.e.m.)

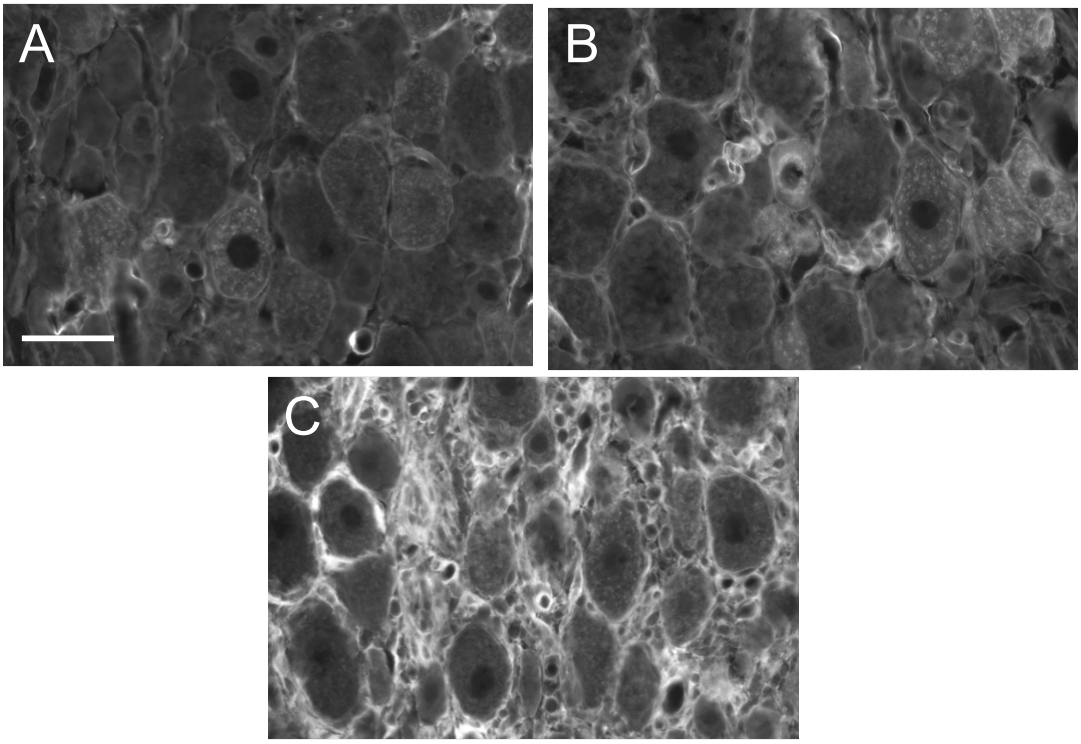

Figure 4.3.2.1 Satellite glial cell p75NTR immunoreactive labeling intensity is dramatically increased with p75NTR AS. Fluorescence photomicrographs of L% DRG sections processed for immunohistochemistry depict p75NTR protein expression in the cytoplasm of SGCs from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) results in increased expression of p75NTR in SGCs compared to control non-infused (A) and p75NTR SC OGN infused (B) treatment groups. Scale bar = 50 μm

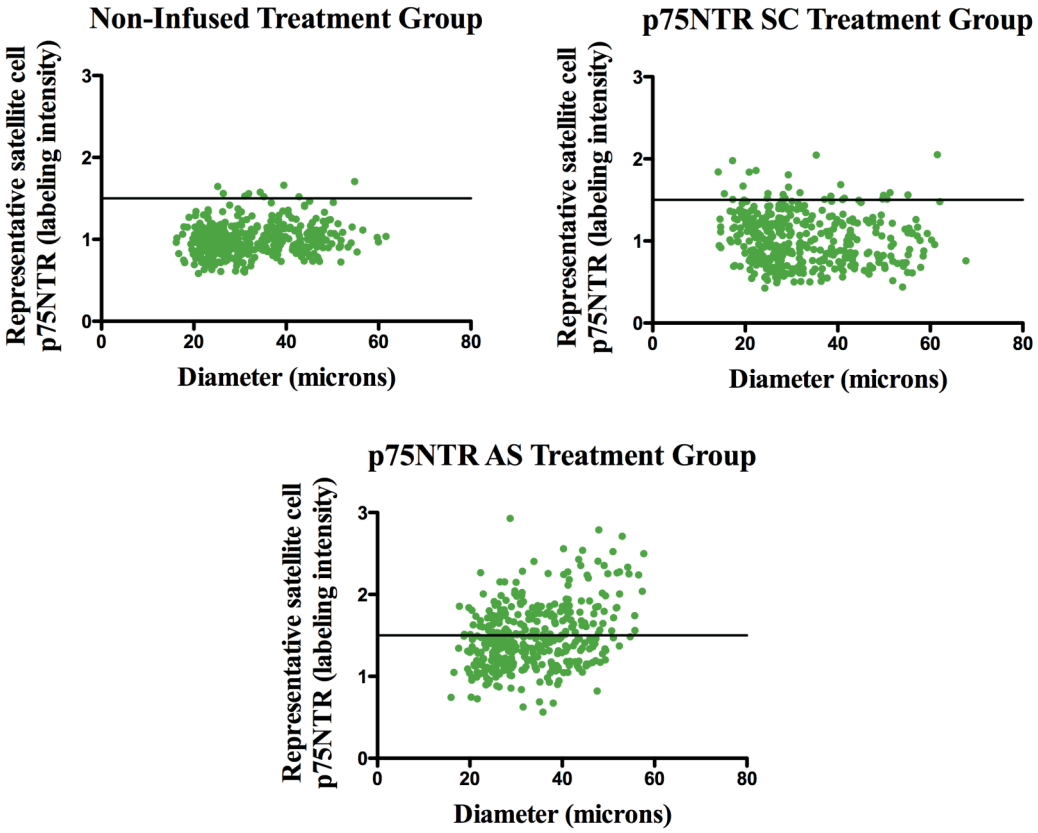

Figure 4.3.2.2 Satellite glial cell p75NTR immunoreactive labeling intensity is increased with p75NTR AS treatment. Scatter plots depict quantification of relative changes in normalized immunoreactive labeling intensity of p75NTR protein expression in SGCs in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean signal labeling intensity in SGCs from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) results in an increase in expression of p75NTR in SGCs compared to non-infused (n=3) and p75NTR SC OGN infused (n=3) treatment groups. N= A total of 385 to 427 SGCs analyzed per treatment group with a total of 3 animals in each group.

respectively) (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.0001$) (Figure 4.3.2.3).

4.3.3 Satellite glial cell $K_{ir}4.1$ expression

Analysis of sections processed for immunohistochemistry to detect glial expression of $K_{ir}4.1$ protein revealed that 7 day intrathecal infusion of p75NTR AS decreased the immunoreactive labeling intensity compared to non-infused and p75NTR SC controls (Figure 4.3.3.1). Each data point was normalized to the mean immunohistochemical labeling intensity from the non-infused control animal in its grouping. The percentage of neurons with surrounding SGCs expressing detectable $K_{ir}4.1$ immunoreactive signal (ie. data points above the mean immunohistochemical labeling intensity of the non-infused control group (>1.00)) decreased from 44.14% (0.01138 s.e.m.) for the non-infused group to 22.83% (0.00884 s.e.m.) for the p75NTR SC infused group and further to 0.59% (0.00641 s.e.m.) for the AS infused control group (Figure 4.3.3.2). Unlike all other markers examined, $K_{ir}4.1$ was the only marker where a significant change was observed in response to sense control infusion. Intrathecal infusion of the sense control, (p75NTR SC), was sufficient to significantly decrease the mean $K_{ir}4.1$ immunoreactive labeling intensity compared to non-infused controls (from 1.0005 +/- 0.01138 (s.e.m.) to 0.8652 +/- 0.00884 (s.e.m.)), however p75NTR AS infusion resulted in a significantly lower mean immunoreactive labeling intensity and greater overeall reducation in signal intensity than the p75NTR SC infusion (0.6009 +/- 0.00641 (s.e.m.) compared to 0.8652 +/- 0.00884 (s.e.m.) respectively (Kruskal-Wallis test with Dunn's Multiple Comparison test, $p<0.0001$) (Figure 4.3.3.3).

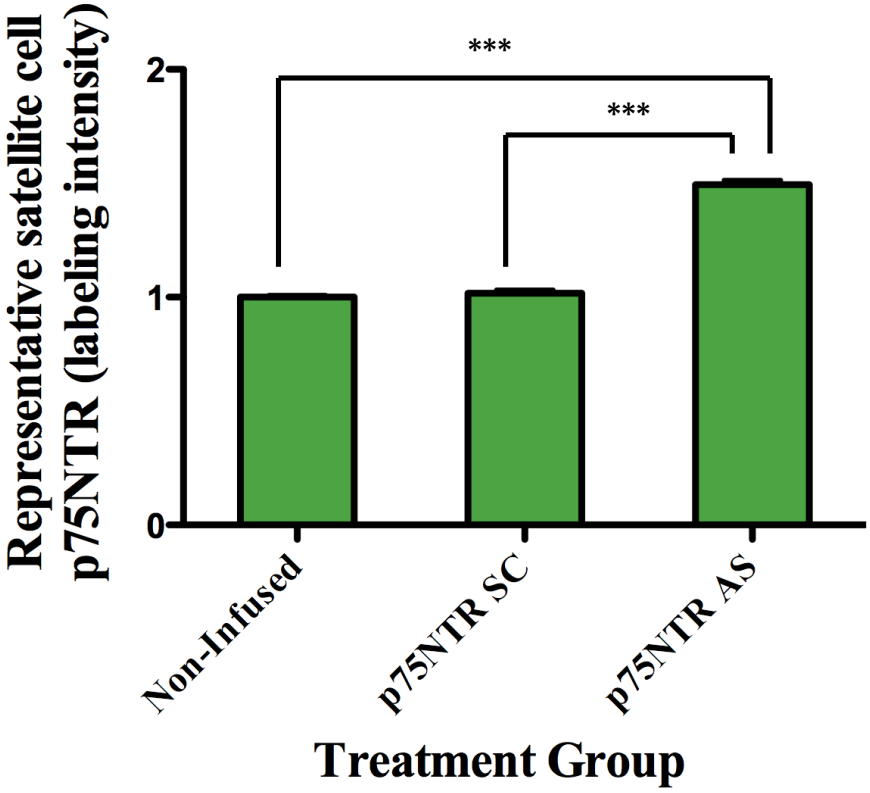

Figure 4.3.2.3 Mean satellite glial cell p75NTR immunoreactive labeling intensity is significantly increased with p75NTR AS treatment. Graph depicts quantification of relative changes in mean immunoreactive labeling intensity of p75NTR protein expression in SGCs in uninjured intact L5 DRG from rats treated as indicated and normalized to the mean labeling intensity in SGCs from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) results in a significant increase in expression of p75NTR in SGCs compared to non-infused (n=3) and p75NTR SC OGN infused (n=3) treatment groups. Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$). Bars represent the standard error of the mean (s.e.m.). N= A total of 385 to 427 SGCs analyzed per treatment group.

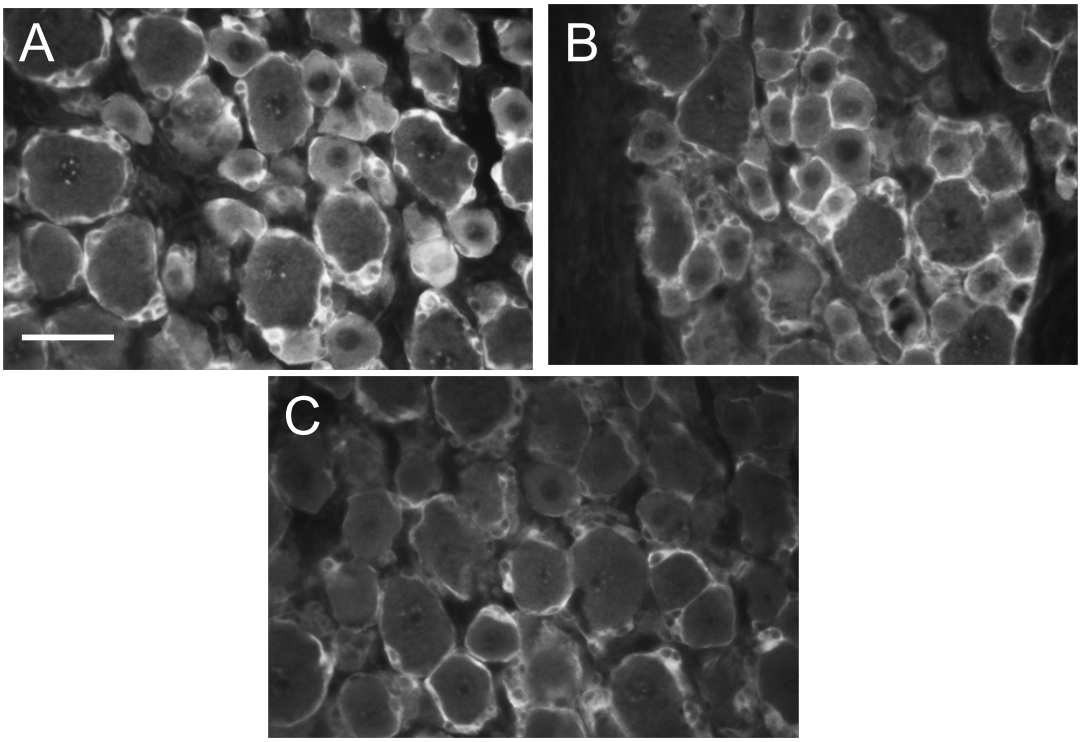

Figure 4.3.3.1 Satellite glial cell K_{ir}4.1 immunoreactive labeling intensity is decreased with p75NTR AS and p75NTR SC treatment. Fluorescence photomicrographs of L5 DRG sections processed for immunohistochemistry depict K_{ir}4.1 protein expression in the cytoplasm of SGCs from uninjured intact rats in control non-infused (A), p75NTR SC infused (B) and p75NTR AS infused (C). Note: Intrathecal infusion of p75NTR AS OGN (C) results in decreased expression of K_{ir}4.1 in both neuronal and SGCs compared to control non-infused (A) and p75NTR SC OGN infused (B) treatment groups. Intrathecal infusion of p75NTR SC OGN (B) also resulted in a modest decrease in K_{ir}4.1 compared to non-infused (A) albeit the mean labeling intensity was significantly higher than the p75NTR AS OGN (C) treated. Scale bar = 50 μm

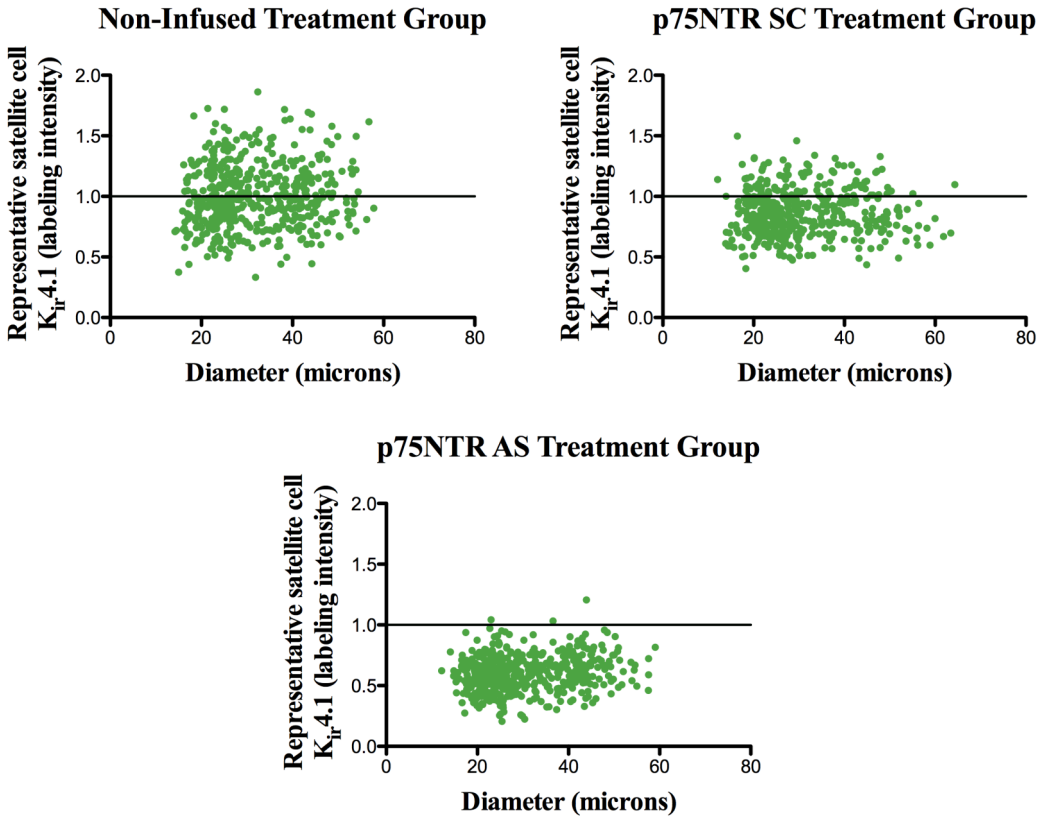

Figure 4.3.3.2 Satellite glial cell $K_{ir}4.1$ immunoreactive labeling intensity is decreased with p75NTR AS and p75NTR SC treatment. Scatter plots depict quantification of relative changes in mean immunoreactive labeling intensity of $K_{ir}4.1$ protein expression in SGCs in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean labeling intensity in SGCs from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) results in a decrease in expression of $K_{ir}4.1$ in SGCs compared to non-infused (n=3) and p75NTR SC OGN infused (n=3) treatment groups. Intrathecal infusion of p75NTR SC OGN also resulted in a decrease in $K_{ir}4.1$ compared to non-infused albeit the immunoreactive labeling intensity was higher than the p75NTR AS OGN treated. N= A total of 460 to 513 SGCs analyzed per treatment group.

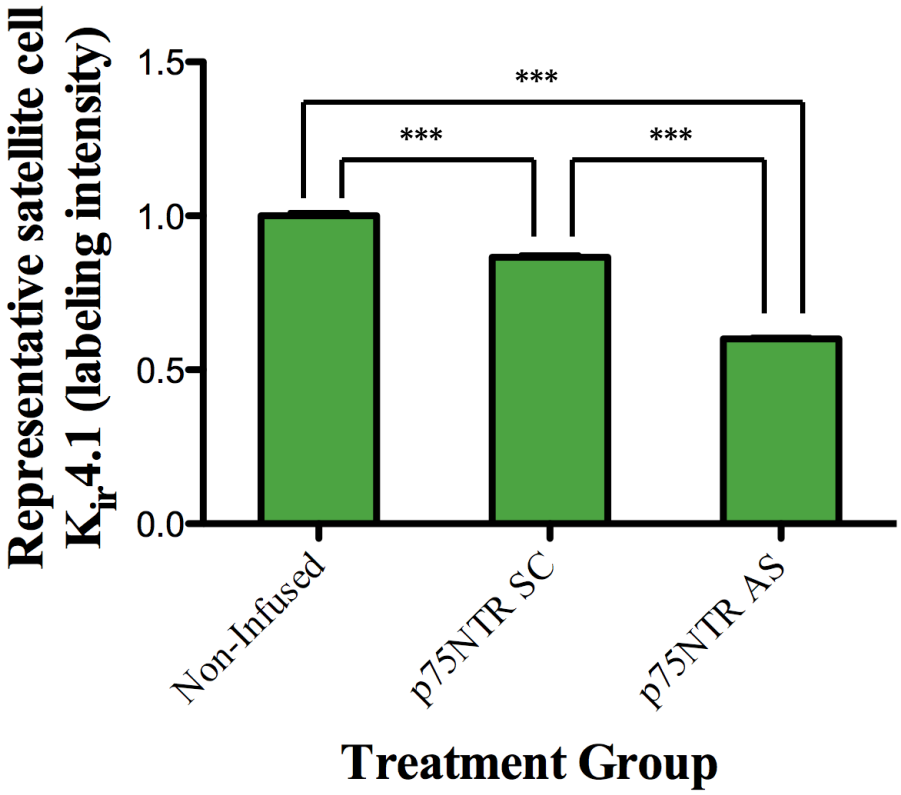

Figure 4.3.3.3 Mean satellite glial cell K_{ir}4.1 immunoreactive labeling intensity is significantly decreased with p75NTR AS and p75NTR SC treatment. Graph depicts quantification of relative changes in mean immunoreactive labeling intensity of K_{ir}4.1 protein expression in SGCs in L5 DRG from uninjured intact rats treated as indicated and normalized to the mean labeling intensity in SGCs from the non-infused control animal in its grouping. Note: Intrathecal infusion of p75NTR AS OGN (n=3) results in a significant decrease in expression of K_{ir}4.1 in SGCs compared to non-infused (n=3) and p75NTR SC OGN infused (n=3) treatment groups. Intrathecal infusion of p75NTR SC OGN also resulted in a significant decrease in K_{ir}4.1 compared to non-infused albeit the mean immunoreactive labeling intensity was significantly higher than the p75NTR AS OGN treated. Asterisks indicate significant differences between experimental groups (Kruskal-Wallis test with Dunn's Multiple Comparison test; *** $p<0.0001$). Bars represent the standard error of the mean (s.e.m.). N= A total of 460 to 513 SGCs analyzed per treatment group.

4.3.4 Satellite glial cell connexin 43 expression

Preliminary results on two sections from two animals from each experimental group showed a qualitative increase in connexin 43, a component of cellular gap junctions, in the SGC region in the p75NTR AS infused treatment group compared to the non-infused control group (Figure 4.3.4). This increase in connexin 43 immunoreactivity was most evident around large size neurons.

4.3.5 Satellite glial cell number increase

Preliminary results showed a quantitative increase in the number of SGCs in the p75NTR AS infused treatment group compared to the non-infused control group (Figure 4.3.5). Preliminary quantitative analysis was performed to determine the average number of SGCs surrounding individual neurons was performed whereby all SGC nuclei surrounding every individual neuron in two sections from two animals from each experimental group were counted then divide by the total number of neurons analysed. This revealed that in the non-infused control group, an average of four to five SGCs surrounding individual neurons was observed. With the infusion of p75NTR AS, an approximate doubling in the number of SGCs surrounding each neuron was observed.

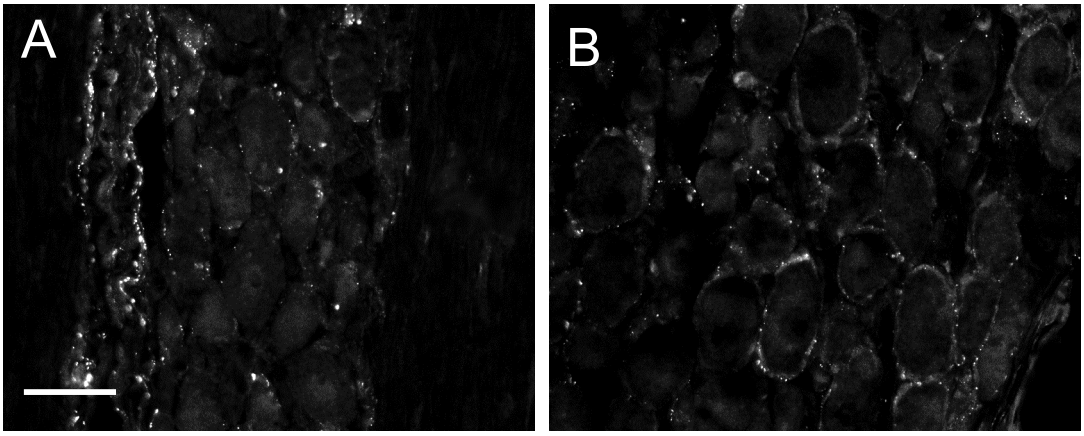

Figure 4.3.4 Satellite glial cell connexin 43 immunoreactive labeling intensity is increased with p75NTR AS treatment. Fluorescence photomicrographs of L5 DRG sections processed for immunohistochemistry depict connexin 43 protein expression in the cytoplasm of SGCs from uninjured intact rats in control non-infused (A) and p75NTR AS infused (B). Note: Intrathecal infusion of p75NTR AS OGN (B) results in increased expression of connexin 43 in SGCs compared to the control non-infused (A) treatment group. Scale bar = 50 μm

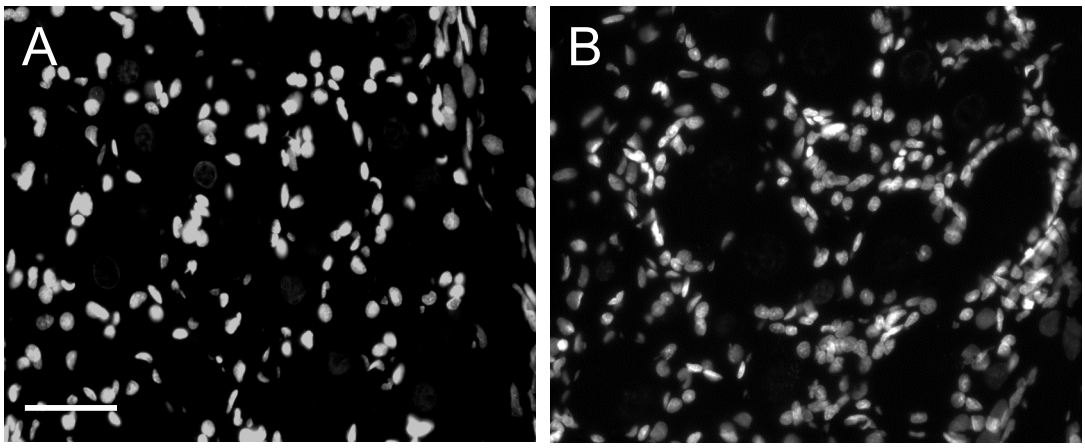

Figure 4.3.5 Satellite glial cell number is increased with p75NTR AS treatment.
Fluorescence photomicrographs of L5 DRG sections labeled with DAPI depict the number of SGCs from uninjured intact rats in control non-infused (A) and p75NTR AS infused (B). Note: Intrathecal infusion of p75NTR AS OGN (B) results in increased number of SGCs compared to the control non-infused (A) treatment group. Scale bar = 50 μm

5. Discussion

5.1 Summary of major findings

Work performed for this thesis revealed that: 1) the intrathecal infusion of p75NTR AS resulted in significantly decreased neuronal p75NTR protein levels; 2) the p75NTR AS uptake in the DRG appeared restricted to only the sensory neurons, not the SGCs; 3) reduced neuronal p75NTR did not result in significantly altered $Na_V1.8$, $Na_V1.9$ or TrkA expression in the DRG; 4) reduced neuronal p75NTR was associated with induction of a 'reactive state' in SGCs consisting of significantly increased expression of GFAP and p75NTR and significantly decreased $K_{ir}4.1$ expression. In addition, preliminary experiments revealed that the altered phenotype induced by p75NTR AS also corresponded with an increase in the number of SGCs surrounding each neuron and an elevated expression of the gap junction protein connexin 43, suggesting that cell-cell connectivity/communication was also altered.

These experiments utilized an intrathecal AS infusion technique previously employed by our laboratory (Wilson-Gerwing et al., 2009). By infusing a small quantity of p75NTR AS over 7 days in the intrathecal space of the spinal cord, its uptake is only visualized in DRG neurons, presumably due to uptake by the axons proximal to the infusion site. The SGCs in the corresponding DRG, appear not to have taken up the p75NTR AS as there was no accumulation of the fluorescent signal in these cells and no reduction in p75NTR protein expression. The confinement of the p75NTR AS to only the neurons was consistent with the distribution that we also observed when we intrathecally administered siRNA (Geremia et al., 2010). Further, this low dose of p75NTR AS over a 7 day period, although sufficient to significantly decrease neuronal cytoplasmic p75NTR

levels in non-injured DRG, appeared to do so without inducing nonspecific/inflammatory effects, as evidenced by a lack of increase in the cell stress marker, ATF-3, and the immunological cell marker, ED-1 (JN, data not shown). Finally, this intrathecal infusion technique has been used by several labs (Barclay et al., 2002; Lai et al., 2002; Obata et al., 2006; Wilson-Gerwing et al., 2009) to deliver a variety of AS to DRG neurons. Consistent with our findings, previous studies employing the same p75NTR AS sequences have noted marked reductions in neuronal p75NTR protein expression as detected immunohistochemically (Obata et al., 2006; Wilson-Gerwing et al., 2009).

Preliminary behavioural testing was performed on these animals to determine whether infusion of p75NTR AS in the absence of a nerve injury was sufficient to alter the latency of paw lifts in a thermal hyperalgesia test. Consistent with a previous report (Obata et al., 2006), no changes were observed in basal pain sensitivity (JN, personal observations).

5.2 Reduced expression of p75NTR in sensory neurons does not significantly impact Na^+ channel nor TrkA expression

Nociception, the ability to sense noxious stimuli, is fundamental to the survival of an organism. When excessive chemical, mechanical or thermal stimuli is applied to tissue, nociceptive nerve fibers depolarize, which initiates action potentials. Maintaining a baseline threshold for action potentials is essential to the accurate transmission of sensory information; if the threshold is decreased, aberrant neural firing may occur, creating a neuropathic pain state. This baseline threshold is contingent on the proper functioning of voltage and proton-gated Ca^{2+}, K^+ and Na^+ ionic channels. Associated

with the transmission of pain and blocked by the commonly known analgesic Lidocaine® are voltage-gated Na^+ channels 1.8 ($Na_V1.8$) and 1.9 ($Na_V1.9$) (reviewed in Blair and Bean, 2002; Priest and Kaczorowski, 2007). These channels are responsible for the majority of the inward action potential current and significant increases in their expression levels, such as that which occurs with injury, alters the neuronal excitability threshold. This hypersensitive state allows for aberrant action potential firing, experienced as chronic neuropathic pain (Blair and Bean, 2002; Lai et al., 2004; Waxman et al., 1999). $Na_V1.8$ and $Na_V1.9$ are highly expressed within the TrkA-expressing nociceptive neuronal subpopulation; over 80% are positive for $Na_V1.8$, and $Na_V1.9$ expression is reported to be limited to only this subpopulation within the DRG (Djouhri et al., 2003a; Fang et al., 2002), as well as in all of the IB4 positive, GDNF-responsive subpopulation (Fukuoka et al., 2008).

We report here that no significant changes in the expression levels of $Na_V1.8$, $Na_V1.9$ or TrkA were observed in the DRG and sciatic nerves when neuronal expression levels of p75NTR protein were significantly decreased for 7 days in intact/non-injured animals by AS treatment. It is of note though that there was a slight shift in the size range of neurons that immunolabel for $Na_V1.8$. It is expressed in all size ranges of neurons in naïve or p75NTR SC treated animals but p75NTR AS treatment results in very few large (>50 mm) being highly immunoreactive for $Na_V1.8$. A similar shift has been observed regarding the TrkA subpopulation following an axotomy (Li et al., 2000). This change in TrkA immunoreactivity distribution was however attributed to a progressive shrinking in neuron size over time. Although little variation was observed after one week, differences were most remarkable at two weeks post-injury. We however failed to

identify many large TrkA-immunopositive neurons regardless of treatment condition. It is thus unknown as to whether this shift in $Na_V1.8$ immunoreactivity was in the subpopulation colocalized with TrkA or without. Investigations with a more prolonged infusion timeline and *in situ* hybridization may elucidate more on this observed size shift in Na^+ channel expression.

Regulation of these Na^+ channels has been postulated to be via different neurotrophic signaling pathways: neurotophins via the Trk receptors, neurotrophins via the p75NTR or GDNF via its own receptor. It is known that maintaining endogenous levels of NGF and GDNF are necessary for preserving $Na_V1.8$ and $Na_V1.9$ neuronal expression levels (Black et al., 1997; Cummins et al., 2000; Dib-Hajj et al., 1998; Fjell et al., 1999). It has been assumed that NGF's actions are via TrkA for regulating Na^+ channel levels, however p75NTR was not considered as an alternative signaling pathway (Fang et al., 2005). Given the results of this study, it suggests that the dominant signaling pathways, in the absence of an injury or inflammation, for homeostatic maintenance of $Na_V1.8$ and $Na_V1.9$ are the NGF/TrkA pathway in the TrkA/p75NTR positive subpopulation and the GDNF/GDNF-α receptor pathway in the IB4 positive/p75NTR negative subpopulation. Thus, decreasing endogenous neuronal p75NTR in the absence of an injury appears not to significantly affect neuronal Na^+ channel levels although it may be that a critical p75NTR reduction threshold had not yet been met.

When introduced in conjunction with a compression nerve injury, p75NTR AS or inhibitory antibody has been shown to diminish hyperalgesia in response to both thermal and mechanical noxious stimuli (Fukui et al., 2010; Obata et al., 2006), supporting the involvement of p75NTR in neuropathic pain states. More research into the interaction

between the various neurotrophins, the Trk receptors and p75NTR with and without an injury is needed to further refine this hypothesis.

5.3 Reduced neuronal p75NTR expression induces a dramatic switch in satellite glial cell phenotype to one of a reactive state

Numerous studies have implicated glial cells of the central nervous system in the promotion and/or preservation of neuropathic pain states (Clark et al., 2007; Coull et al., 2005; Guo et al., 2007). In contrast, very little is known about the glial cells of the peripheral nervous system and their involvement in the neuropathic pain syndrome. Recent findings have implicated a glial cell type unique to the peripheral nervous system, the SGC's, in neuropathic pain states resulting from peripheral nerve injuries. SGCs tightly encapsulate the cell bodies of sensory neurons within the DRG and are thus optimally positioned to help maintain a homeostatic environment, influencing neuronal excitability. The SGCs buffer the perineuronal area and correspondingly express K^+ channels $Kir_{4.1}$ and SK_3 (Hibino et al., 1999; Vit et al., 2006), Ca^{2+}-binding S100 proteins (Sandelin et al., 2004; Vega et al., 1991), glutamate transporters (Berger and Hediger, 2000; Hosli and Hosli, 1978), glutamine synthetase (Miller et al., 2002; Weick et al., 2003) and gap junction proteins permitting the passage of K^+ and other small molecules between adjacent SGCs (Huang et al., 2005; Konishi, 1996; Weick et al., 2003). With a peripheral nerve injury or inflammation, changes have been observed in some of these molecules as well as others. The SGCs become 'reactive' and express higher levels of GFAP, p75NTR, the gap junction constituent, connexin 43, and lower levels of $K_{ir}4.1$ (Ohara et al., 2008; Tang et al., 2010; Zhou et al., 1996). The SGCs also undergo marked

morphological changes with injury; they become hypertrophic, they proliferate and they increase their coupling with one another via gap junctions (reviewed in Pannese, 1981).

Our findings demonstrate a very robust induction of a 'reactive-like' state in the SGCs, in the absence of an injury or inflammation, in response to a significant reduction in neuronal p75NTR protein. This 'reactive-like' state is reminiscent of an injury phenotype; the SGCs expressed significantly higher protein levels of GFAP, p75NTR and significantly lower levels of $K_{ir}4.1$ protein. Preliminary qualitative observations also show an increase in the number of SGCs around each neuron and an increase in connexin 43 protein following neuronal p75NTR AS treatment. This glial reactivity was also induced in the Schwann cells of the sciatic nerve with an observed increase in GFAP. Therefore, the data presented in this thesis demonstrate that neuronal signaling via neuronal p75NTR appears to be necessary to maintain a homeostatic, 'non-activated' state in the SGCs. It is thus demonstrated that the reduction of neuronal p75NTR modulates the sensory neuron/SGC communication axis. The signaling complex responsible for this phenomenon is currently unknown.

Nerve injuries or inflammation also promote the infiltration of immune cells into the perineuronal area that morphologically resemble SGCs (Hu and McLachlan, 2003; van Velzen et al., 2009). Thus, it is possible that some of the observed perineuronal cells may also be resident immune cells (Lu and Richardson, 1993) as the SGC population has not been rigorously defined, however the markers (GFAP, $K_{ir}4.1$, connexin 43, etc) localized to this area are consistent with the majority being glial cells. In addition, no apparent increase in the ED1 (marker for activated macrophages) was observed following p75NTR AS infusion (data not shown) suggesting that an inflammatory response was not

induced. Despite this, it cannot be ruled out that a minor portion of the proliferating cells may be resident immune cells, although this was not determined in my studies.

5.3.1 Sensory neuron/glial communication

Very little is currently known regarding sensory neuron/SGC communication. It is assumed that these cells communicate, given the symbiotic relationship they appear to maintain, however limited evidence of this liaison has emerged to date. The first observation of this phenomenon was described via chemical signaling involving nitric oxide, released by the neurons in response to *N*-methyl-*D*-aspartate receptor activation, which initiated the release of cyclic guanosine 3′,5′-monophosphate from the SGCs (Magnusson et al., 2000; Meller et al., 1992; Thippeswamy and Morris, 1997). A parallel upregulation of nitric oxide synthase (NOS) in neurons and cyclic guanosine 3′,5′-monophosphate in the SGCs directly after axotomy supports Thippeswamy and Morris' (1997) hypothesis of nitric oxide acting as a neuron-glial signaling molecule, possibly promoting neural survival (Shi et al., 1998). However, it is unlikely that nitric oxide is responsible for the SGC 'reactivity' resulting from reduced neuronal p75NTR in these sets of experiments as only approximately 10% of sensory neurons express NOS in a non-injured state (Shi et al., 1998). However, this rapidly increases to 35% two days then 45% one week after peripheral nerve injury (Verge et al., 1992). Whether increased neuronal expression of NOS resulted from the p75NTR AS treatment was not examined.

Since then, in addition to further research on the nitric oxide communication axis, SGCs have been shown to respond to neuronal releases of adenosine triphosphate (ATP) in several different ways. The first is the activation of P_2X ionotropic and/or P_2Y

metabotropic ATP purinoreceptors (Kobayashi et al., 2005, 2006). Their activation can commence calcium-mediated depolarizations amongst SGCs, propagated via their gap junctions (Suadicani et al., 2010). The second is the neuronal release of ATP, stimulating SGCs to also release their own ATP and tumour necrosis factor α, both exerting an excitatory effect on the sensory neurons (Zhang et al., 2007). Injured tissue releases ATP that can initiate an 'activation' of purinoreceptors (reviewed in Burnstock and Wood, 1996), whereas with the p75NTR AS infusion, this factor is missing. The mode of neuron/glial communication responsible for the observed changes in SGC properties in this thesis remains to be elucidated.

5.3.2 Satellite glial cell activation

The intrathecal infusion of p75NTR AS resulted in a very robust induction of a 'reactive-like' state, evidenced by GFAP immunoreactivity, in the SGCs of the DRG. This reactivity was greatest in the SGCs surrounding neurons that did not label for IB4, which by definition, expressed p75NTR (McMahon et al., 1994; Wright and Snider, 1995). In the DRG, I noted that SGCs express very low levels of GFAP under normal conditions in agreement with Vit et al. (2006). Following an injury or chronic inflammation, GFAP levels steadily increase until most SGCs are GFAP-immunoreactive (Siemionow et al., 2009). This increase in GFAP correlated with an increase in SGC p75NTR immunoreactivity. Perineuronal cell p75NTR expression is also upregulated following a nerve injury (Zhou et al., 1996), but is normally expressed at low levels under nonpathological conditions (Obata et al., 2006).

In addition to changes in GFAP and p75NTR in the perineuronal area, an increase in TrkA protein expression was observed in what appeared to be fine axonal processes. SGCs have not been reported to express TrkA mRNA, however sympathetic nerve axons have detectable trkA protein (Martin-Zanca et al., 1990; Tessarollo et al., 1993).

These TrkA positive structures resembled rings around some of the neuronal cell bodies. Sympathetic nerve axons will sprout around the cell bodies of nociceptive neurons positive for TrkA, in 98% of cases, forming basket-like structures in response to nerve injuries (McLachlan et al., 1993; Walsh et al., 1999). It has been observed that these basket-like formations are closely associated with increased SGC p75NTR levels and SGC neurotrophin expression (Zhou et al., 1999; Zhou et al., 1996). It has been shown that sprouting sympathetic axons follow myelinated sensory axons and wrap around the SGCs, but are not directly juxtaposed to the neuronal cell body (Ramer and Bisby, 1998, 1999) perhaps as a result of tropic gradients set up by the increased expression of neurotrophins in the reactive SGCs. Of note, their sprouting is reduced in p75NTR knockout mice (Ramer and Bisby, 1997) perhaps because the perineuronal cells do not induce the same reactive state. Thus, it appears that the reduction of neuronal p75NTR may have indirectly induced sympathetic nerve axon sprouting by upregulating expression of neurotrophins in conjunction with the other markers of the reactive state, although this was not ascertained.

With such a significant increase in the cytoskeletal protein, GFAP, akin to an injury state, we postulated that the SGCs may be proliferating and increasing their coupling, as it has been shown that SGCs undergo changes in cell coupling and gap junction protein expression levels following a nerve injury (Gunjigake et al., 2009).

Although we did not look at coupling directly, preliminary results revealed an increase in the mean number of SGCs around each neuronal cell body and an increase in the gap junction protein, connexin 43, which has previously been localized to astrocytes and SGCs (Nagy et al., 1997; Ohara et al., 2008). It has been postulated by some that the ratio of SGCs per neuronal cell body is regulated by the metabolic requirements of that neuron (Pannese, 1981; Zimmermann and Braun, 1999). Thus, the infusion of p75NTR AS may be altering the metabolic function of the sensory neurons that in turn, requires the activation and proliferation of SGCs to cope with these changes.

5.3.3 Satellite glial cells as therapeutic targets for pain control

The most insightful experiments undertaken to investigate the role of SGCs in the regulation of pain pathways have employed the use of AS, RNA interference (RNAi) or silencing RNA (siRNA) technology. These techniques involve reducing or near complete silencing of a molecule of interest in non-injured/intact animal models. These investigations have provided solid data supporting roles for $K_{ir}4.1$ and connexin 43 in SGCs in regulating neuropathic pain states and may help in identifying novel therapeutic targets for pain control.

The silencing of connexin 43 in the SGCs by RNAi has been investigated by Ohara et al. (2008), Procacci et al. (2008) and Vit et al. (2006). It appears that the behavioural response following the injection of connexin 43 double stranded RNA depends on whether a nerve injury is present or not: pain-like behaviour was reduced after a nerve compression and pain-like behaviour was induced in the absence of an injury (Ohara et al., 2008). Thus, it appears that connexin 43 expression levels, if not

kept within an optimal range, can significantly contribute to the development of a neuropathic pain state. We report that the significant reduction of neuronal p75NTR induced an upregulation of connexin 43 in SGCs within the DRG, albeit no behavioural consequences were observed upon preliminary examination of nociceptive thresholds (JN, personal observations). It is possible that more effective silencing of neuronal p75NTR protein production with the use of RNAi or siRNA might produce a more robust upregulation of connexin 43 to the point of inducing pain-like behaviour (Ohara et al., 2008) or alternatively additional factors not expressed in the current experiments are required for this response. Another molecule of interest, $K_{ir}4.1$ has been associated with neuropathic pain. Vit et al. (2008) reported that an injection of double stranded RNA (RNAi technology) targeting $K_{ir}4.1$ into the trigeminal nerve, in the absence of a nerve injury, was sufficient to induce pain-like behaviour in rats. The outcome of introducing $K_{ir}4.1$ RNAi post-injury is currently unknown. We report that the significant reduction of neuronal p75NTR induced a downregulation of $K_{ir}4.1$ in SGCs within the DRG, albeit no behavioural changes were observed. It is possible that more effective silencing of neuronal p75NTR with the use of RNAi or siRNA, in the same case as connexin 43, may significantly downregulate $K_{ir}4.1$ expression and induce pain-like behaviour. Unlike all other markers examined, $K_{ir}4.1$ was the only marker where a significant change was observed in response to p75NTR SC infusion. $K_{ir}4.1$ has been described as highly sensitive to extracellular K^+ concentration fluctuations (reviewed in Olsen and Sontheimer, 2008). Its expression pattern, spatially and temporally, in SGCs, positively correlates with neuron size (Durham and Garrett, 2010). Proper uptake of glutamate in astrocytes is most effective at a negative resting potential, a parameter directly contingent

on K_{ir}4.1 functioning (Kucheryavykh et al., 2007), suggesting a similar role in SGCs. Thus, this may reflect K_{ir}4.1 and SGCs' ultra sensitivity to any perturbation of the homeostatic state within the sensory system.

In conclusion, the findings in my thesis support a critical role for neuronal p75NTR signaling in the maintenance of the homeostatic state in sensory neurons with respect to SGCs transition to a reactive state associated with a number of pathologies. SGCs are now being recognized as key modulators of neuropathic pain states and are thus potential therapeutic targets (Capuano et al., 2009). Elucidating the messengers responsible for the communication axis between the SGCs and their sensory neurons and the factors that transition these cells from normal to pathological states will aid in understanding the mechanism of neuropathic pain and the generation of novel therapeutics.

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